Identification and pathogenicity of *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae)

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There is a dim light at the other end of the tunnel
The tunnel brightens more as I draw closer
Fear, my worst enemy wants me to give up
Hope my most valuable companion keeps me moving on
Taking one step ahead is not easy but I shall overcome
For I believe that all the suffering is not put to waste
For there is a dim light at the other end of the tunnel

-Nathan Gwira-

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IDENTIFICATION AND PATHOGENICITY OF *BURSAPHELENCHUS* SPECIES (NEMATODA: PARASITAPHELENCHIDAE)

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR (PHD) IN APPLIED BIOLOGICAL SCIENCES

HONGMEI LI

IDENTIFICATIE EN PATHOGENICITEIT VAN *BURSAPHELENCHUS* SPECIES (NEMATODA: PARASITAPHELENCHIDAE)

COVER FIGURE: PINE WILT DISEASE IN PURPLE MOUNTAIN, NANJING, CHINA IN 1985.

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Chapter 1

General Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 and its vector insects, cerambycid beetles belonging to the genus *Monochamus* Dej., 1821, have been found associated with pine wilt disease (PWD) in South Korea, Taiwan, Japan and China (Mamiya & Enda, 1972; Tzean & Jan, 1985a; Yang & Wang, 1989; La *et al*., 1999). The nematode has also been detected in pine wood in the USA, Canada and Mexico (Dropkin & Foudin, 1979; Knowles *et al.*, 1983; Dwinell, 1993). *Bursaphelenchus xylophilus* represents a great threat to the forestry industry and is a major plant quarantine objective for most countries in the world. Despite the measures taken by the European and Mediterranean Plant Protection Organization (EPPO) and the European Union (EU) to prevent the accidental introduction of *B. xylophilus* with imported wood products from infested areas, *B. xylophilus* was discovered in Portugal in 1998 (Mota *et al.*, 1999). The threat from *B. xylophilus* has led the EU to restrict the import of pine wood products from North America, Japan, Korea and China and other regions of Asia. This has had serious economic consequences.

In China, pine wilt disease was first discovered in 1982 on Japanese black pine, *Pinus thunbergii* Parl., 1868, in Nanjing, Jiangsu Province (Cheng *et al*., 1983). The incidence of pine wilt disease requires a minimum summer temperature of no lower than 20^{o}C (Rutherford *et al*., 1990) and global climate change has greatly increased areas of central and southern China in which the temperatures suitable for pine wilt disease occur. Consequently, pine wilt disease is becoming an increasingly serious problem in these regions (Yang, 2004). Since China joined the World Trade Organization (WTO) in 2001, increased international trade has increased the risk of introducing exotic insects and diseases. Most of the shipping containers used to package imported and exported goods contain wood, usually from unprocessed lower quality hardwood or coniferous wood. Such materials are more likely to harbour dangerous pests, such as *B. xylophilus* and/or its vectors. It has been proven that PWN can be spread over long distances *via* the wood used for the packing of shipped commodities (La *et al.*, 1999; Suzuki, 2004; Yang, 2004). PWN has been frequently intercepted from imported wood packaging in China (Gu *et al.*, 2006a). A number of phytosanitary regulations (directive 77/93 updated as 2000/29/EC) have been implemented by EU countries to prevent the introduction of *B. xylophilus* and its vectors from the infected countries. Like other governments, the Chinese government also implemented quarantine regulations that restricted coniferous packing wood imported from the USA, Japan, Korea and the European Union from year 2000. Under the terms of these restrictions all imported wood packaging materials have to be inspected and sampled by national Entry-exit Inspection and Quarantine Bureaus.

There are two pathways for transmission of *B. xylophilus*. Long distance transmission occurs as a result of human activities in which nematodes are transported to uninfected areas in dead trees or logs from infected regions. International timber trading also can introduce the nematodes into uninfected countries. Wood packaging materials used in shipping containers may also transfer the nematodes between countries. In the natural condition PWN is transmitted over relatively short distances by *Monochamus* spp. The nematodes are transferred as fourth-stage dispersal juveniles which are found throughout the tracheal system of beetles (Enda, 1994; Aikawa & Togashi, 2000), The nematodes can invade new host trees *via* feeding or oviposition wounds made by adult beetles (Mamiya & Enda, 1972; Linit, 1988; Edwards & Linit, 1992; Arakawa & Togashi, 2002). The transmission of nematodes by the beetles is one of the factors determining the epidemiology of disease (Jikumaru & Togashi, 2001). Once inside a suitable host and in appropriate conditions, tree mortality may result within a few months. Mortality caused by PWN is associated with the presence of highly susceptible host species and high temperatures (Yang, 2004). The dependence on temperature means that mortality rates vary depending on the time of year in which the host is infected; plants infected early in the summer may die as soon as 40 days after infection whereas infections in spring may take longer to cause death (Kiyohara & Tokushige, 1971). Limited information is available about the responses of the tree to biotic stresses including PWN, a reflection of the paucity of information available on tree-pathogen and pathogen-vector interactions more generally.

There are no effective control measures for PWN, except intensified quarantine, clear cutting and replanting. Plant quarantine by border or post-border inspections for packaging wood or dead pine trees is considered the most effective measure to prevent the introduction and distribution of the pine wood nematode (Braasch *et al.*, 2004a). The clear cutting and replanting are currently practiced in PWN infested countries in East Asia. The treatment of clear-cut dead pine trees may include methyl bromide fumigation, hot water treatment, chipping the dead wood, high temperature treatment, submerging in water and burying in soil. Direct trapping of vector adults in terpene-baited traps or aerial and ground sprays of sumithion insecticides can significantly reduce tree death rates by killing the beetle vectors. In China, these methods do not give effective control in the large areas infested by PWN. Millions have been spent to control the spread of the disease, but with only limited success so far. PWN remains the number one forest pest and poses a devastating threat to pine forests in southern and central China (Yang, 2004). Despite several decades of efforts in Japan to control the nematode and the insect vectors, the disease has been spread throughout the entire

country, with the exception of Aomori and Hokkaido (Mamiya, 2004; Shimazu, 2006). In recent years a continuous spread of the disease has been observed in new different areas in South Korea and PWN has caused enormous damage to native pine stand and became the major forest pest in South Korea (Shin & Han, 2006).

The most effective method for PWN control is the use of resistant *Pinus* species or other coniferous species. However, effective resistance depends on the aggressiveness of the nematode populations and their pathogenicity to plants (Ikeda, 1984). Pathogenicity tests of *Bursaphelenchus* species to *Pinus* species have been intensively investigated under both field and laboratory conditions and often yield results that vary between different reports. Seedling tests, which are among the most commonly used tests, seem to be particularly variable. Pathogenicity studies with *Bursaphelenchus* species are extremely difficult to perform accurately due to the difficulties of simulating natural inoculation of beetle vectors. Species that are rarely (or not) pathogenic in nature are often recorded as pathogenic using experimental inoculation methods. McNamara (2004) criticized the techniques used in the reported pathogenicity tests. These tests gave very variable results and the experiments reported to date do not give any confidence in the relevance of the results in relation to field conditions. There is obviously an urgent need for in-depth research into the criteria which determine the response of coniferous plants after inoculation with nematodes in relation to various factors, such as age of host plant, method of inoculation (including use of vectors to deliver inoculum), state of sterility of the nematode suspension, life stage of the nematodes, environmental conditions, *etc*. in order to find a means to relate such experiments to natural conditions of infection and disease expression (McNamara, 2004).

Despite the damage caused by *B. xylophilus* throughout the world, little is clearly known about the molecular mechanisms underlying nematode pathogenicity. The few molecular studies that have been carried out to date have focused on the development of diagnostic tools rather than examining mechanisms of pathogenicity. Identifying candidate genes related to pathogenicity and virulence of the nematode will provide breeding strategies to develop pine varieties with broad and durable resistance, and thus contribute greatly to the efficient management of the pine wilt disease. This non-chemical management of the disease will bring a sustainable and healthy control and benefit to the environment and public health.

The objectives of this study related to the long-time goal of efficiently managing pine wilt disease in China and elsewhere are:

1. Identifying *Bursaphelenchus* species from packaging wood materials from different countries imported to Nanjing by morphological, morphometrical and molecular

methods, to inspect the distribution of a potential pathogenic population from infected area to non-infected area through national or international trade and decrease the economic consequences by preventing the nematode's introduction through plant quarantine (Chapter 4).

2. Monitoring the emergence of adult JPS from wilt-killed *P. thunbergii* logs and examining PWN transmission to pine twigs through maturation feeding, to better understand the dynamics of pine wilt disease initiation in Nanjing, China (Chapter 5).
3. Evaluating the effect of various factors to the pathogenicity of *B. xylophilus* on *P. thunbergii* by different inoculation tests including simulating natural inoculation of *B. xylophilus* through beetle vectors, to establish a comparable and accurate method for evaluating the pathogenicity of *B. xylophilus* on pine species and proposing the standard for estimating the reproductive isolated populations to be a potential pathogenic nematode (Chapter 6).
4. Screening pathogenic and less-pathogenic *B. xylophilus* populations using the established method on *P. thunbergii* and three other *Pinus* species, to identify variation within pathogenicity genes (from an EST database) associated with differences in pathogenicity (Chapter 6-7).
5. Establishing the optimized conditions required for dsRNA to operate in *B. xylophilus* and knocking out putative pathogenicity related genes by RNAi to *in vitro* analyse the function of candidate genes related to pathogenicity of *B. xylophilus*. Developing and applying molecular tools to analyse function of selected pathogenicity genes in *B. xylophilus* (Chapter 7).

Chapter 2

Bursaphelenchus species: biology, identification and pathogenicity

The economic importance of PWN has generated extensive research work on *Bursaphelenchus* species and their related insect vectors. Priority areas have included studies on the identification and phylogenetic relationships of *Bursaphelenchus* species, the development and improvement of diagnostic techniques and the exploration of host, plant and insect vector interactions. This review chapter summarizes and analyses recent developments in these fields and provides comprehensive taxonomic information for the *Bursaphelenchus* genus in general and *B. xylophilus* in particular.

2.1 Pine wood nematode *Bursaphelenchus xylophilus*

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, vectored by cerambycids belonging to the genus *Monochamus*, is the causal agent of the pine wilt disease. PWN originated in North America and has been introduced to some East Asian countries and to Portugal. It is a dangerous forestry pest which plays an important role in world and national economies. The distribution, biology and control of *B. xylophilus* are summarised here.

2.1.1 History and distribution

B. xylophilus was first described in the USA as *Aphelenchoides xylophilus* Steiner & Buhrer, 1934. Later it was described as *Bursaphelenchus lignicolus* when it was determined to be the causal agent of pine wilt disease in Japan (Mamiya & Kiyohara, 1972). The synonymy was recognized in 1981 (Nickle *et al*., 1981).

B. xylophilus is considered to be indigenous to North America and is widespread in natural coniferous forests which mostly consist of resistant pine species (Robbins, 1982; Rutherford *et al.*, 1990; Bowers *et al.*, 1992; Sutherland & Peterson, 1999). Any significant losses caused by *B. xylophilus* in North America are recorded almost exclusively among exotic species and in artificial forest ecosystems (Evans *et al.*, 1996). The possible explanation is that both plant and nematode have co-evolved for a very long time in North America and thus the trees have become resistant/tolerant to the presence of the nematodes (Kiyohara & Bolla, 1990). However, when *B. xylophilus* reached the non-native habitats in East Asia, where local trees had no previous exposure to the pathogen the situation changed dramatically and caused a disaster for local forestry. Some landmarks of pine wood nematode research are listed in Table 2.1.

Table 2.1 Landmarks in the history of pine wood nematode research.

Year	Event	Reference
1905	First report on pine wilt disease in Nagasaki, Kyushu island, Japan	Yano, 1913
1929	*Aphelenchoides xylophilus* found in association with fungi in timber	Steiner & Buhrer, 1934
1969	*Bursaphelenchus* sp. found in wood of dead pine trees in Kyushu, Japan	Tokushige & Kiyohara, 1969
1970	*Aphelenchoides xylophilus* transferred to genus *Bursaphelenchus*	Nickle, 1970
1971	*Bursaphelenchus* sp. demonstrated as the causal agent of pine wilt disease by inoculation tests on *Pinus* spp.	Kiyohara & Tokushige, 1971
1972	Pine wood nematode described as *B. lignicolus* Transmission of *B. lignicolus* through maturation feeding of *Monochamus alternatus* reported	Mamiya & Kiyohara, 1972 Mamiya & Enda, 1972
1979	*B. mucronatus* described from *P. densiflora* in Japan *B. lignicolus* found in *P. nigra* and *P. sylvestris* in Missouri, USA	Mamiya & Enda, 1979 Dropkin & Foudin, 1979
1981	*B. lignicolus* placed as a synonym of *B. xylophilus*	Nickle *et al*, 1981
1982	*B. xylophilus* found in *P. thunbergii*, in Nanjing, China	Cheng *et al*, 1983
1983	Transmission of *B. xylophilus* during oviposition of *Monochamus* vectors reported *B. xylophilus* found in Canada	Wingfield &. Blanchette, 1983 Knowles *et al*, 1983
1984	*B. xylophilus* intercepted in pine wood chips imported into Finland from the United States and Canada	Rautapää, 1986
1985	*B. xylophilus* found in *P. luchuensis* and *P. thunbergii* in Taiyuan and Taoyeun prefecture, Taiwan	Tzean & Jan, 1985a; 1985b
1989	*B. xylophilus* found in *P. densiflora* and *P. thunbergii* in Pusan, South Korea	Yi *et al.*, 1989
1993	*B. xylophilus* found in *P. estevesii* in Mexico	Dwinell, 1993
1998	*B. xylophilus* found in *P. pinaster* in Peninsula of Setúbal, Portugal	Mota *et al.*, 1999

B. xylophilus is assumed to have been transported to Japan by means of contaminated wood products from USA at the beginning of the 20th century (Yano, 1913; Nickle *et al*., 1981; Mamiya, 1983; Malek & Appleby, 1984). The nematode was confirmed as the causal agent of pine wilt disease (Kiyohara & Tokushige, 1971) and shown to be vectored by *Monochamus alternatus* (Mamiya & Enda, 1972). Although several decades of efforts have been put in to trying to control the nematode and the insect vectors, the disease has spread throughout Japan, with the exception of the most Northern prefectures of Aomori and Hokkaido (Mamiya, 2004; Shimazu, 2006).

PWN was also dispersed to other East Asian countries. In China, the first discovery of PWN was reported on *P. thunbergii* in Nanjing 1982 (Cheng *et al*., 1983) and assumed to be introduced through infected wood materials. More than 20 years continuous spread of PWN across six provinces resulted in millions of trees being killed. The forest landscape and ecology were faced with a devastating threat (Britton & Sun, 2002). Appropriate conditions for PWN establishment are present in vast areas of central and southern China. The potential dispersion of pine wilt disease is becoming serious in these regions (Yang, 2004). In Taiwan, the first occurrence of the disease was reported by Tzean and Jan (1985a). Since then the disease has been present throughout northern Taiwan and has caused serious damage to forests covering almost half of the entire island. In South Korea, PWN was first detected by Yi *et al*. (1989) and was thought to be introduced from Japan in infested wood used for packaging imported goods. The PWN infected areas were restricted to a relatively small region in the south of the country and the distribution of the disease was controlled until 1997 (La *et al.*, 1999). However, in recent years spread of the disease has been observed to new areas of South Korea. PWN has caused enormous damage to native pine stands and has become the major forest pest in South Korea (Shin & Han, 2006).

PWN was recently discovered on *P. pinaster* in Portugal (Mota *et al.*, 1999), the first description of *B. xylophilus* in Europe. Subsequent genetic studies traced the introduction as arising from East Asian countries (Metge & Burgermeister, 2006; Vieira *et al*., 2007). After the initial detection in 1998, a national survey was carried out and a quarantine area (encompassing the PWN infected area) and safety buffer area were established. The latest survey revealed a significant increase in the number of declining trees within the infected zone and resulted in an expansion of the delimited area. New prevention measures were implemented by the EU which meant that all the pine trees were cut and removed from the 3 km phytosanitary strip surrounding the quarantine area by the end of 2007 (Rodrigues, 2006). The permanent establishment of PWN in Portugal could become a serious problem for the European timber industry unless it is eradicated or its spread restricted (Braasch, 2000b). The EU has restricted the import of pine wood products from North America, Japan, Korea and China and other regions of Asia.

2.1.2 Biology

The biology of *B. xylophilus* is synchronized with the development of the vector *Monochamus* spp. The PWN is transported as fourth-stage dispersal juveniles to new host

trees by cerambycid beetles through their maturation feeding wounds, or to dead or dying trees during beetle oviposition. After the tree dies, the nematodes feed on and reproduce on various wood-inhabiting fungi (Kobayashi *et al.*, 1974; Kobayashi *et al.*, 1975; Fukushige, 1991; Kuroda & Ito, 1992).

2.1.2.1 Life cycle and behaviour

The life cycle of *B. xylophilus* has two development phases, phytophagous (transmission by feeding) and mycophagous (transmission by oviposition) (Wingfield, 1983) (Fig. 2.1).

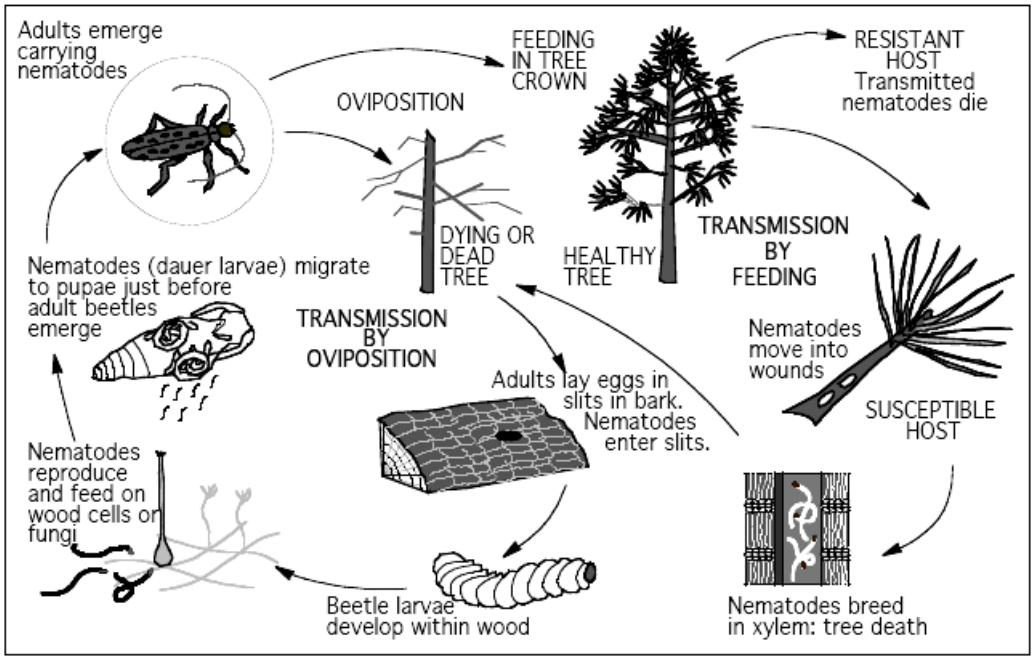

Fig 2.1 Schematic representation of the inter-relationships between the pinewood nematode, *Bursaphelenchus xylophilus*, and its vectors in the genus *Monochamus* (adapted from Fielding and Evans, 1996).

Transmission during maturation feeding is the initiation of the phytophagous phase of the nematode, which has the greatest importance for the potential development of pine wilt disease. *Monochamus* larvae develop initially in the cambium of the tree and then burrow into the wood in order to make a pupal chamber. While the pupa develops, third-stage dauer juveniles of *B. xylophilus* congregate in the vicinity of the pupal chambers, possibly under the influence of substances diffusing from the pupa (Mamiya, 1983). When the adult beetle is due to emerge, the nematodes moult to the fourth-stage dispersal juvenile and gather at the tips of the long-necked perithecia of fungi which surround the pupal chambers and project into the chamber. The nematodes are picked up when newly emerged beetles brush against the perithecial necks. The fourth stage (dauer-like) juveniles settle under the elytra and in the

tracheal system of the beetle (Enda, 1994) and migrate into the tree through the wounds caused by feeding (Mamiya & Enda, 1972; Linit, 1988; Linit, 1990; Yang *et al*., 2002) or oviposition of vector (Wingfield & Blanchette, 1983; Edwards & Linit, 1992; Arakawa & Togashi, 2002).

Many studies on the phytophagous phase have been carried out. Emerging beetles fly to fresh *Pinus* plants and feed on the bark of twigs. During this maturation feeding episode, the juvenile nematodes leave the insect and enter the plant through the feeding wounds made by beetles (Kobayashi *et al.*, 1984). Exodus of juveniles from the beetle occurs mainly between 10 and 40 days after beetle emergence (Togashi & Sekizuka, 1982). After being introduced to a suitable tree species and under favourable climatic conditions, the nematode multiplies quickly on pine parenchymal cells (Togashi & Sekizuka, 1982) then migrates from the cambium into the xylem. The nematodes cause death of the tree due to blocking water conductance through the xylem and by reducing oleoresin exudation. Dead trees show the classical symptoms of pine wilt disease (Giblin-Davis, 1993). The dying or dead trees are colonized by blue stain fungi (usually *Ceratocystis* spp.) which provide a further food source for the nematodes and became attractive to vector beetles which gathered on the trunk to mate and lay eggs (Fauziah *et al.*, 1987; Togashi, 1989a).

Transmission during oviposition leads to the mycophagous phase. When the female beetles lay their eggs in recently killed or dying trees or in cut logs with bark, the fourth stage dispersal juveniles migrate out of the beetle's tracheal system and enter the tree through the oviposition slits in the bark made by female beetles. Immediately after entering the wood the dauer nematodes moult to the adult stage and begin laying eggs. The nematodes in wood propagate rapidly if suitable fungi are present in the trees. The nematode population will cease to multiply and begin to decline under stress conditions and the special dispersal third-stage dauer juveniles are formed which are capable of resisting adverse conditions (Mamiya, 1983). The presence of fungi in wood encourages nematode reproduction and survival and, consequently, increases the number of nematodes carried by emerging beetles (Mamiya, 1984; Linit, 1988; Kishi, 1995; Fielding & Evans, 1996).

Bursaphelenchus xylophilus can be maintained on fungal cultures in the laboratory. It reproduces in 12 days at 15°C, 6 days at 20°C and 3 days at 30°C. Egg-laying starts on the 4th day after hatching and the eggs hatch in 26-32 h at 25°C. The temperature threshold for development is 9.5°C (Futai, 1980).

2.1.2.2 Host plants

Bursaphelenchus xylophilus can be associated with several conifer species; however, it is mainly found in pine species (*Pinus* spp.). The nematode can only infect a limited number of living *Pinus* species and these can be killed as mature trees in natural conditions. The nematode susceptible species include the Far Eastern species *P. bungeana, P. densiflora, P. luchuensis, P. massoniana a*nd *P. thunbergii*, which are widely planted in their native habitats, the European species *P. nigra* and *P. sylvestris* planted in North America and *P. pinaster* planted in Portugal and China (Duncan & Moens, 2006). Many other *Pinus* species have been found to be damaged or killed by inoculation with *B. xylophilus* (mainly as seedlings in glasshouses) (McNamara, 2004).

Other coniferous species can also act as hosts of *B. xylophilus* including Atlas cedar and Deodara cedar (*Cedrus* spp.), Eastern larch, European larch (*Larix* spp.) and Balsam fir (*Abies* spp.) (Robbins, 1982; Bowers *et al.*, 1992). However, these species are rarely reported to be killed by the nematodes. Outside the genus *Pinus,* only the death of Blue spruce (*Picea* pungens) and Douglas fir (*Pseudotsuga menziesii*) in the USA have been reported relating to the nematode (Malek & Appleby, 1984).

2.1.2.3 Insect vectors

As mentioned above, *B. xylophilus* is transported as fourth-stage dispersal juveniles to new host trees mainly by vector cerambycid beetles of the genus *Monochamus*. These insects only oviposit on recently felled trees or trees already under stress and their larvae can cause economic losses by forming bore holes in the wood. However, *Monochamus* spp. has a far greater economic impact in countries where *B. xylophilus* is present.

Monochamus species that can act as the vectors of *B. xylophilus* occur only in the northern hemisphere. *Monochamus alternatus* was the major vector of *B. xylophilus* in several East Asian countries (Japan, China, South Korea and Taiwan) where the nematode is present (Mamiya & Enda, 1972; Yi *et al*., 1989; Yang, 2004; Chang *et al*., 1995). In Europe *M. galloprovincialis* has been confirmed to transmit *B. xylophilus* in Portugal (Sousa *et al*., 2001). *Monochamus carolinensis*, *M. mutator*, *M. scutellatus* and *M. titillator* are the vector insects for *B. xylophilus* in North America (Dropkin *et al.*, 1981; Linit 1988; Edwards & Linit, 1992), of which *M. carolinensis* and *M. scutellatus* are the major vectors. *Monochamus alternatus* was the most efficient vector of *B. xylophilus* when compared to the North American beetles

(Linit *et al*., 1983). The numbers of nematodes carried by beetles were investigated on *M. alternatus* (Lee *et al.*, 1990; Jiang *et al*., 2002, Li *et al*., 2007) and *M. carolinensis* (Linit *et al.*, 1983; Malek & Appleby, 1984).

Other genera of the Cerambycidae (e.g. *Acalolepta*, *Acanthocinus*, *Amniscus*, *Arhopalus*, *Asemum*, *Corymbia*, *Neacanthocinus*, *Rhagium*, *Spondylis*, *Uraecha*, *Xylotrechus*) and other Coleoptera (e.g. *Chrysobothris*, *Hylobius*, *Pissodes*) have been found to carry *B. xylophilus* in or on their bodies but their roles as vectors in nature still need further investigation (Ryss *et al*., 2005). Many of the *Monochamus* spp. from conifers have also been reported as being associated with non-pathogenic *Bursaphelenchus* spp.

2.1.2.4 Movement and dispersal

Bursaphelenchus xylophilus can move within the wood tissues which it infects and may be found from the top of the branch to the end of root of the host plant. However, the nematodes are incapable of moving from one host tree to another without their vectors. The natural spread of *B. xylophilus* among host plants depends on the natural activities of the vector. The beetles have been recorded as capable of flying for up to 3.3 km, but in most cases the dispersal distance is only a few hundred metres (Kobayashi *et al*., 1984). Therefore, the travelling distance of beetles largely limits the spreading distance of wilt disease each year if no artificial transportation of logs and wood products infested with beetles and the nematodes are involved (Takasu *et al*., 2000).

Infested wood is the most important pathway for international spread of *B. xylophilus* and the nematode has been intercepted on sawn wood, round wood, wood chips and packaging wood (Rautapää, 1986; Braasch *et al.*, 2001; Gu *et al.*, 2006a). Wood packaging material constructed from unprocessed coniferous wood infected with nematodes is thought to be the most likely means of distributing *B. xylophilus* during international trade (Evans *et al*., 1996), especially when the nematodes are imported together with vector insects which can carry the nematodes to new coniferous trees (McNamara & Stoen, 1988).

The long-term establishment of *B. xylophilus* into a new region requires the nematode to find a means of contact with a native vector in spite of whether it is introduced with or without a vector insect (Duncan & Moens, 2006). The introduction and dispersal of *B. xylophilus* into new areas is also assisted by the high phenotypic plasticity of the nematode, including the ability to overcome resistance of host trees and the ability to survive prolonged periods in transit (i.e. long periods of starvation) (Mamiya, 1984).

2.1.3 Management and Control

Because *B. xylophilus* has caused serious damage to coniferous forests in several countries in the Far East and is a putative pathogen for European forests, it is listed as an A1 quarantine pest by EPPO (OEPP/EPPO, 1986). Control measures against pine wilt disease (PWD) aim at breaking the pine tree-nematode-insect disease triangle. So far it has proved impossible to control *B. xylophilus* once introduced into a tree or to eradicate PWD once introduced into a country (Nakamura & Yoshida, 2004). The prevention of the spread of the nematode remains the basis of management programs using such techniques as intensified plant quarantine, felling, de-barking and burning of damaged pines as well as chemical control of vector beetles (Suzuki, 2004).

A number of phytosanitary regulations have been implemented, including an EU directive (77/93 updated as 2000/29/EC), to prevent the introduction of *B. xylophilus* and its vectors from countries where the nematodes occur. Wood products must be heated or chemically treated in order to kill *Monochamus* and *Bursaphelenchus* as a legal requirement prior to importation to some countries (Webster, 1999). Heat treatment is known to be an effective and environmentally acceptable method for wood infected with *B. xylophilus* and its vectors. Heat processing by kiln drying the wood materials at 56°C for 30 minutes can completely kill the vector insect and *B. xylophilus* (Duncan & Moens, 2006).

PWD has severely damaged pine forests in Japan and a great deal of effort has been made to prevent the spread of the disease within the country. These efforts focus mainly on the combination of removing dead or dying trees from the forest to prevent their use as a source of further infection and control of vector beetles with insecticides. Large amounts of money have been spent on removal of diseased trees and aerial-spraying of chemicals (mostly fenitrothion) to kill the insect vectors (Ikeda, 1984). The removal of dead trees from the forest will reduce their use by beetles for laying eggs and thus prevent them from being a reservoir for further distribution. The aerial spraying of insecticides can minimize the transmission of *B. xylophilus* by adult beetles. For protecting individual pine trees from PWN infection, nematicide injection into tree bodies has been developed (Matsuura, 1999). Recently, avermectin has been applied to control *B. xylophilus* in *P. thunbergii* and *P. massoniana* by trunk injection (Lin & Zhou, 2004).

Many other attempts have been made to develop alternative measures for control of PWD, including biological control of nematodes and vectors (Shimazu *et al*., 1995; Xu *et al.*,

2002; Lai *et al*., 2002; Shimazu, 2004), use of insect attractants (Jiang *et al*., 1998; Zhao *et al*., 2000a), breeding of resistant *Pinus* clones and inducing resistance by inoculation of non-pathogenic strains of *B. xylophilus* (Kiyohara *et al.*, 1999; Kosaka *et al.*, 2001). However, these approaches have met with limited success to date.

2.2 Taxonomy and identification of *Bursaphelenchus* species

2.2.1 Taxonomical position of genus *Bursaphelenchus* (De Ley & Blaxter, 2004)

Kingdom: Animalia
Phylum: Nematoda
Class: Chromadorea
Order: Tylenchida
Suborder: Aphelenchina
Superfamily: Aphelenchoidoidea
Family: Parasitaphelenchidae
Genus: *Bursaphelenchus*

2.2.2 The species of genus *Bursaphelenchus*

The genus *Bursaphelenchus* was established by Fuchs (1937) and currently comprises around 90 described species. The type species is *B. piniperdae* Fuchs, 1937. This genus has been studied in detail for many years owing to its economic importance; the discovery of PWN in Portugal in 1998 further increased the worldwide interest in this genus. For example, an analysis of the number of new *Bursaphelenchus* species described shows several peaks in the number of new species. The first occurred shortly after the description of the genus, another after the identification of *B. xylophilus* as the causal agent of pine wilt disease followed by a more recent increase subsequent to the discovery of *B. xylophilus* in Portugal (Fig. 2.2).

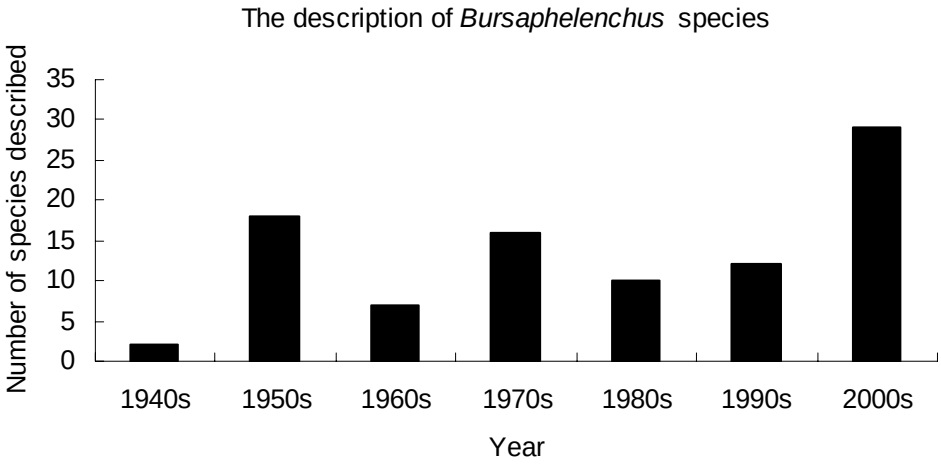

Fig 2.2 The chronology of species description of genus *Bursaphelenchus.*

The genus *Bursaphelenchus* is mainly distributed in the northern hemisphere and is associated with plantations of pine trees (Ryss *et al.*, 2005). Only two species, *B. cocophilus* from coco plant and *B. africanus* from imported packaging wood from South Africa (Braasch *et al*., 2007a), have been described from outside of this geographical range. Most species of *Bursaphelenchus* are associated with insects and dead or dying coniferous trees (Fig 2.3) and are mycetophagous (Hunt, 1993). Most vectors of *Bursaphelenchus* spp. are forest Coleoptera and examples include scolytid bark beetles, cerambycid longhorn beetles and nitidulid beetles.

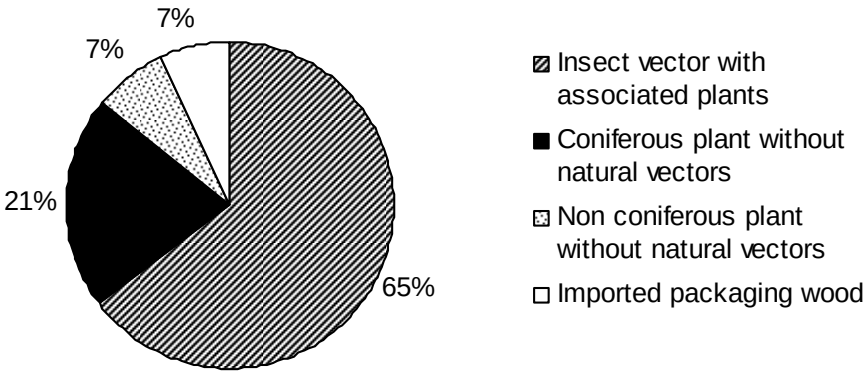

Fig 2.3 The relationship between *Bursaphelenchus* species and natural vectors and associated plants.

Thorough investigations into the species composition, distribution and associated plants of *Bursaphelenchus* have been carried out in some west European countries (Tomminen *et al.*, 1989; Braasch *et al.*, 2000; Braasch, 2001; Braasch & Philis, 2002; Abelleira *et al.*, 2003; Braasch, 2004a; Peñas *et al.*, 2004). Recently the description of new species and the reports of the species diversity have increased dramatically (Dan & Yu, 2003; Tomiczek *et al.*, 2003; Braasch, 2004b; Palmisano *et al.*, 2004; Gu *et al.*, 2006a). Twenty-nine species of *Bursaphelenchus* have been described since 1999 and these are listed in Table 2.2. The information for *Bursaphelenchus* described before this date is described in Ryss *et al.* (2005). Thirteen of these were described from dead wood of dead or dying trees of *Pinus* spp., nine from insect organs and seven were described from packaging wood intercepted as a result of quarantine measures.

Recent studies have suggested that some *Bursaphelenchus* species other than *B. xylophilus* may be pathogenic to young pines under particular circumstances (Kulinich *et al*., 1994; Kishi, 1995; Braasch, 1996; Mamiya, 1999; Braasch *et al.*, 2000; Braasch, 2001; Michalopoulos-Skarmoutsos *et al.*, 2004). However, besides *B. xylophilus*, only *B. cocophilus* within the genus is recognised as being an important pest in agriculture and forestry. *Bursaphelenchus cocophilus*, known as the red ring nematode, is vectored by palm weevils and devastates palm trees and coconut plantations in Caribbean islands and Central and South America (Cobb, 1919; Dean, 1979; Esser & Meredith, 1987; Gerber & Giblin-Davis, 1990; Griffith & Koshy, 1990; Araújo *et al.*, 1998; Brammer & Crow, 2001; Harrison & Jones, 2003).

Table 2.2 *Bursaphelenchus* species described after 1999 with their natural vectors and associated plants.

Year	Species	Country	Associated plant	Insect vector	Reference	Place of nematode isolated
2000	*B. abietinus*	Austria (Vorarlberg, Bludenz)	*Abies alba* Mill. (Pinales: Pinaceae)	*Pityokteines curvidens* (Germar), *P. spinidens* (Reitter), *P. vorontzovi* (Jacobson) (Coleoptera: Scolytidae)	Braasch & Schmutzenhofer, 2000	Fir bark beetles
	B. conicaudatus	Japan (Kyoto)	*Ficus carica* L. (Urticales: Moraceae)	*Psacothea hilaris* (Pascoe) (Coleoptera: Cerambycidae)	Kanzaki *et al*., 2000	Yellow-spotted longicorn beetle
	B. paracorneolus	Germany (Potsdam)	*Picea abies* (L.), *Pinus sylvestris* L. (Pinales: Pinaceae)		Braasch, 2000c	
2002	*B. aberrans*	China (Guangdong Province)	*Pinus massoniana* Lamb. (Pinales: Pinaceae)		Fang *et al*., 2002a	Dead wood
	B. dongguanensis	China (Guangdong Province)	*Pinus massoniana* Lamb., (Pinales:Pinaceae)		Fang *et al*., 2002b	Dead wood
	B. rainulfi	Malaysia (Peninsular Malaysia)	*Pinus caribaea* Morelet. (Pinales: Pinaceae)		Braasch & Burgermeister, 2002	Dead wood
	B. thailandae	Thailand	*Pinus merkusi* Jungh & de Vriese (Pinales: Pinaceae)		Braasch & Braasch-Bidasak, 2002	Dead wood
2003	*B. baujardi*	India (Haryana)	*Bombax ceiba* L. (Malvales: Bombacaceae)		Walia *et al*., 2003	Insect frass on trunk of the tree
	B. luxuriosae	Japan	*Aralia elata* (Miq.) (Apiales: Araliaceae)	*Acalolepta luxuriosa* Bates (Coleoptera: Cerambycidae)	Kanzaki & Futai, 2003	The udo longicorn beetle
	B. minutus	India (Himachal Pradesh)	*Pinus wallichiana* AB Jackson (Pinales: Pinaceae)		Walia *et al.*, 2003	Dead wood

Year	Species	Country	Associated plant	Insect vector	Reference	Place of nematode isolated
2004	*B. doui*	Taiwan and South Korea (wood intercepted in China)	*Pinus* sp. (Pinales: Pinaceae)		Braasch *et al*., 2004a	Imported wood packaging
	B. fuchsi	Russia (Primorsky territory)	*Pinus koraiensis* Sieb. & Zucc. (Pinales: Pinaceae)		Kruglik & Eroshenko, 2004	Dead wood
	B. lini	China (Nanjing)	*Pinus massoniana* Lamb., *P. thunbergii* Parl. (Pinales: Pinaceae)		Braasch, 2004b	Dead wood
	B. sinensis	China (wood intercepted in Austria)	*Pinus* sp. (Pinales: Pinaceae)		Palmisano *et al*., 2004	Imported wood packaging
	B. vallesianus	Switzerland (Stalden, Canton Valais)	*Pinus sylvestris* L. (Pinales: Pinaceae)		Braasch *et al*., 2004b	Dead wood
2005	*B. anatolius*	Turkey (Ankara)	*Onopordum turcicum* Danin (Asteraceae).	*Halictus* sp. (Hymenoptera: Halictidae)	Giblin-Davis *et al.,* 2005	Soil dwelling bee
	B. arthuri	Taiwan and South Korea (wood intercepted in China)	*Pinus* sp. (Pinales: Pinaceae)		Burgermeister *et al*., 2005a	Imported wood packaging
	B. singaporensis	Singapore (wood intercepted in China)	non-coniferous hardwood		Gu *et al.*, 2005	Imported wood packaging
2006	*B. antoniae*	Portugal (Leiria)	*Pinus pinaster* Aiton (Pinales: Pinaceae)	*Hylobius* sp. (Coleoptera: Curculionidae)	Peñas *et al.*, 2006	Large pine weevil
	B. gerberae	Trinidad (Manzanilla)	*Cocos nucifera* L. (Arecaceae: Cocosoideae)	*Rhynchophorus palmarum* L. (Coleoptera: Curculionidae)	Giblin-Davis *et al.*, 2006a	Palm weevil
	B. hildegardae	Germany (Markendorf, Brandenburg)	*Pinus sylvestris* L. (Pinales: Pinaceae)	*Hylurgops palliates* Gyllenhal (Coleoptera:Scolytidae)	Braasch *et al.*, 2006	Pine wood and insect

Year	Species	Country	Associated plant	Insect vector	Reference	Place of nematode isolated
2006	*B. platzeri*	USA (California)		*Carpophilus humeralis* (Coleoptera: Nitidulidae)	Giblin-Davis *et al.*, 2006b	Pineapple beetle
	B. willibaldi	Germany (Beeskow, Brandenburg)	*Pinus sylvestris* L. (Pinales: Pinaceae)		Schonfeld *et al*., 2006	Wood chips
	B. yongensis	China (Zhejiang Province)	*Pinus massoniana* Lamb. (Pinales: Pinaceae)		Gu *et al*., 2006b	Dead wood
2007	*B. africanus*	South Africa (wood intercepted in China)	*Pinus radiato* D. Don (Pinales: Pinaceae)		Braasch *et al*., 2007a	Imported wood packaging
	B. anamurius	Anamur-Mersin and Bergama-Izmir in Turkey	*Pinus brutia* Ten. (Pinales: Pinaceae)		Akbulut *et al*., 2007	Dead wood
	B. burgermeisteri	Japan (wood intercepted in China)	*Pinus radiato* D. Don. (Pinales: Pinaceae)		Braasch *et al*., 2007b	Imported wood packaging
	B. uncispicularis	China (Yunnan Province)	*Pinus yunnanensis* (Pinales: Pinaceae)		Zhuo *et al.*, 2007	Dead wood
2008	*B. chengi*	Taiwan (wood intercepted in China)	*Pinus* sp. (Pinales: Pinaceae)		Li *et al.*, 2008	Imported wood packaging

2.2.3 The taxonomy of the genus *Bursaphelenchus*

Much effort has been put into subdividing the large number of described *Bursaphelenchus* species into smaller sub-groups using different criteria. Tarjan & Baéza-Aragon (1982) were the first to propose spicule morphology as the primary diagnostic character for the species of *Bursaphelenchus.* Subsequently, Giblin and Kaya (1983) used spicule shape to separate several groups within *Bursaphelenchus* and Yin *et al.* (1988) constructed a key to species in the genus *Bursaphelenchus* according to the spicule characteristics. Braasch (2001) established nine different species groups in the genus based mainly on the number of lateral lines, followed by the number and arrangement of the male caudal papillae, spicule shape, presence of the vulval flap in the female and the shape of female tail. However, these characters cannot be used for all described species because some of the characters are unavailable for some species. This classification system therefore has limited utility for identification.

Ryss *et al.* (2005) attempted to construct an integrated morphological identification system for all the species of the genus. All species in the genus were separated into 6 groups based solely on spicule characters. The six species groups (*aberrans*-group, *borealis*-group, *eidmanni*-group, *hunti*-group, *piniperdae*-group and *xylophilus*-group) are classified as identification units for the purpose of facilitating species identification. However, while some of these groups could be considered as natural based on their phylogenetic relationship (e.g. the *xylophilus*-group), some of them could be considered as artificial. For example, the *piniperdae*-group contains species with two, three or four lateral lines and various arrangements of caudal papillae. Consequently this grouping of *Bursaphelenchus* species was challenged by Lange *et al.* (2007) and Ye *et al. (*2007) who suggested that the phylogeny of the *Bursaphelenchus* species should combine molecular data with morphological characters, especially spicule shape and the number of lateral lines.

Despite the problems with the grouping system proposed by Ryss *et al*., (2005) noted above, the members of the *xylophilus*-group can be clearly separated from other groups based solely on the male spicule characters and this group is therefore likely to be robust. However, the variation in, and overlapping range of, several other taxonomic characters within some species of this group makes accurate identification to species level difficult. Within the *B. xylophilus* group, species can be distinguished by the shape of the female tail. *Bursaphelenchus xylophilus* is distinguished from other species in the genus by a rounded tail shape without a distinct mucron (R-Form). However, female *B. xylophilus* from North

America (M-Form) show variations in tail shape from rounded to a mucronated form making it extremely difficult to separate it from *B. mucronatus*, a non-pathogenic species in which the female has a mucronate tail (Wingfield *et al.*, 1983). The morphological similarities between *B. xylophilus* and *B. mucronatus* females coupled to the fact that some samples consist solely of males or juvenile stages mean that identification to the species level within the *xylophilus*-group is extremely difficult (and frequently unreliable) using morphological data alone (Bolla & Wood, 1999; Braasch, 2004a).

2.2.4 Species identification within *Bursaphelenchus*

The economic importance of PWN means that there is a need for accurate identification of species from the genus *Bursaphelenchus.* In particular it is important to be able to rapidly and accurately identify certain species for quarantine purposes. Different methods have been used for the identification and diagnosis of *Bursaphelenchus* spp.

2.2.4.1 Morphological methods

Analysis of morphological characters remains the standard method for routine identification of the species in the genus *Bursaphelenchus*. Microscopical observation is recognised as the basic approach for identification of species but an experienced taxonomist is needed in order to distinguish *Bursaphelenchus* species from other nematodes. Morphometric measurements under the microscope can provide substantial amounts of information used for species identification. In addition, scanning electron microscopy (SEM) can provide more detailed information, such as the number of incisures in the lateral field, the spicule shape and the arrangement of caudal papillae, which are considered as important diagnostic features of *Bursaphelenchus* species. Microscope based approaches are still considered the most important tools used for the identification of *Bursaphelenchus* species (Coomans, 2002). However, variation in, and the overlapping range of some morphological characters makes identification to the species level difficult for some members of the *xylophilus*-group, including the economically important *B. xylophilus* and *B. mucronatus.* Therefore, alternative methods for identifying these species based on protein and DNA analysis have been developed.

2.2.4.2 Protein based methods

A method was developed for distinguishing four *Bursaphelenchus* species by analysis of the protein profiles revealed by SDS-PAGE (Hotchkin & Giblin, 1984). Enzyme

electrophoresis (analysed after isoelectric focusing – IEF) was applied to distinguish *B. xylophilus* and *B. mucronatus* isolates on the basis of enzyme staining patterns (Guiran *et al.*, 1985; Hu & Yang, 1995). Immunological methods have also been developed for species-specific identification. Polyclonal antibodies were produced that differentiate specific antigens of certain *B. xylophilus* isolates on Western Blots (Lawler *et al*., 1993). Monoclonal phage antibodies (Fonseca *et al.*, 2006) and monoclonal antibodies (Jiang *et al.*, 2006) have also been prepared that identify *B. xylophilus* isolates. Although the methods above have aided the identification of nematode species, their value is limited by differential gene expression in nematode stages during the life cycle or responses to external environmental influences. DNA-based methods, however, offer a more reliable alternative as the DNA profile of a species should be consistent regardless of life stage or environment (Harmey & Harmey, 1993).

2.2.4.3 DNA based approaches

DNA-based methods have been widely used in taxonomic studies, analysis of genetic variation and analysis of phylogenetic relationships among *Bursaphelenchus* spp.. The genomic DNA, ribosomal DNA and mitochondrial DNA have been analysed by different methods for the purposes of specific and intraspecific identification.

2.2.4.3.1 DNA-hybridization methods

These methods are based on specific hybridization of a radioactively or non-radioactively labelled probe to target DNA. Total genomic DNA can be analysed for species differentiation by using restriction enzymes followed by hybridization of DNA probes (RFLP). Bolla *et al.* (1988) differentiated *B. xylophilus* isolates and *B. mucronatus* using RFLP analysis. Other probes have been used in similar studies including the unc-22 gene of *C. elegans* (Abad *et al.*, 1991), ribosomal DNA (Webster *et al.*, 1990), homologous DNA probes (Tàres *et al*., 1992; Harmey & Harmey, 1994) and satellite DNA (Tàres *et al.*, 1994) to differentiate isolates of *Bursaphelenchus* spp. within *xylophilus*-group. However, these approaches are of limited utility for species identification due to the need for large quantities of pure DNA. In addition, the techniques used in these approaches are too complex and expensive to make them attractive for routine analysis of nematode samples.

2.2.4.3.2 PCR-based methods

The development of the polymerase chain reaction (PCR) promoted the establishment of

a variety of new molecular biology methods which require only small amounts of DNA. Amplification and analysis of specific regions of DNA has proved to be an effective approach for the identification and the phylogenetic analysis of inter- and intra-specific variation in the genus *Bursaphelenchus*.

RAPD-PCR uses an arbitrary primer for random amplification of fragments of any part the genome and can generate polymorphic DNA patterns. RAPD-PCR has been used for analysis of intra-specific variation in PWN isolates from China (Zheng *et al.*, 1998; Zhang *et al.*, 1999; Zhang *et al*., 2006), Japan (Kusano *et al.*, 1999), and a mixture of different geographical isolates (Braasch *et al.*, 1995; Irdani *et al.*, 1995; Wang *et al.*, 2001; Zhang *et al.*, 2002). RAPD-PCR studies have also been used to study genetic relationship among 30 isolates of *B. xylophilus* from the native regions and non-indigenous areas (Metge & Burgermeister, 2006) and 24 Portuguese *B. xylophilus* isolates (Vieira *et al*., 2007).

More work has been concentrated on the analysis of specific genomic regions, such as the ribosomal DNA (rDNA) gene family, mitochondrial DNA (mtDNA) and satellite DNA, for diagnostic and phylogenetic analysis.

The rDNA gene family is a multigene family (Fig. 2.4), which consists of many copies (100 to 500 in animals) of genes encoding three ribosomal RNA subunits 28S, 5.8S and 18S, interrupted by the internal and external transcribed (ITS and EST) and intergenic (IGS) non-coding spacers. ITS regions which are highly conserved within species have received the most attention for the identification of *Bursaphelenchus* species.

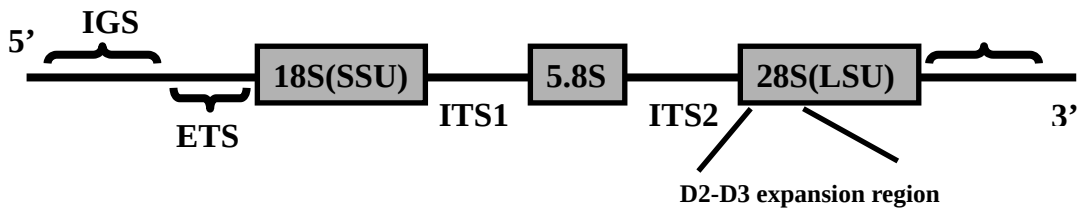

Fig 2.4 Structure of the ribosomal DNA gene family in nematodes. Coding regions of the 18S small subunit (SSU), 5.8S and 28S large subunit (LSU), non-coding regions of internal transcribed spacers (ITS1 and ITS2), external transcribed spacer (ETS) and the intergenic spacer (IGS).

Restriction length polymorphisms of ITS regions (ITS-RFLP) have been used for the identification of *Bursaphelenchus* species (Iwahori *et al.*, 1998; Hoyer *et al.*, 1998; Liao *et al*., 2001; Zheng *et al.*, 2003). The ITS-RFLP patterns produced by digestions of ITS regions with five restriction endonucleases (*Rsa*I, *Hae*III, *Msp*I, *Hinf*I and *Alu*I) was suggested as a tool for *Bursaphelenchus* species identification and used to support description of new species (Burgermeister *et al.*, 2005b; Giblin-Davis *et al.*, 2005; Peñas *et al*., 2006). Specific

detection of *B. xylophilus* has been achieved by designing specific primer pairs based on ITS sequences followed by standard PCR (Kang *et al.*, 2004; Li *et al.*, 2004; Leal *et al.*, 2005), or improved PCR techniques, such as PCR-SSCP (Zhang *et al*., 2001), duplex PCR (Zhao *et al.*, 2004; Matsunaga & Togashi, 2004), nested-PCR (Takeuchi *et al*., 2005) and real-time PCR assays (Cao *et al.*, 2005; Leal *et al*., 2007). The sequence of rDNA ITS regions also have been used in the assessment of the relationships among isolates with different geographical origins (Iwahori *et al.*, 1998; Beckenbach *et al.*, 1999; Kanzaki & Futai, 2002a; Megte *et al.*, 2006; Lange *et al.*, 2007). The sequence analysis of the D2D3 domain of the 28S rDNA (LSU) has proven useful in species diagnosis within confusing groups (De Ley *et al*., 1999). Other studies have demonstrated the utility of LSU for phylogenetic analysis of *Bursaphelenchus* species (Ye *et al*., 2007).

mtDNA sequences of animals evolve at a faster rate compared to the nuclear genes and have been useful for discriminating closely related *Bursaphelenchus* species. These studies have analysed the mitochondrial cytochrome oxidase subunit I (COI) gene (Beckenbach *et al.*, 1999; Kanzaki & Futai, 2002b; Zhang *et al.*, 2002; Iwahori *et al.*, 2004). The sequencing of a heat shock protein gene, *hsp*70 (Beckenbach *et al*., 1992) and satellite DNA-based PCR amplification (Abad, 2000; Castagnone *et al.*, 2005, François *et al.*, 2007) have also been developed for the purpose of identification and phylogenetic analysis of *Bursaphelenchus* species.

In general, routine methods for precisely identifying *Bursaphelenchus* species need a combination of morphological and morphometric characters assisted by molecular analysis of ITS-RFLP patterns. The phylogeny studies based on the sequence of D2D3 of 28S rDNA data are useful for genetic research of *Bursaphelenchus* species, but also need to refer to the morphological characters. The morphological-molecular approach is strongly recommended for species descriptions and diagnosis of the genus *Bursaphelenchus*.

2.3 Pathogenicity of *Bursaphelenchus xylophilus*

The development of symptoms after *B. xylophilus* infection and the incidence of pine wilt disease are related to the interactions between host species, environmental conditions, nematode populations and other microorganisms. The mechanism of pathogenicity of *B. xylophilus* is reviewed here to clarify the pathology of this complicated disease.

2.3.1 Development of disease

During nematode-induced death of pine two phases of symptom development occur after invasion of the wood by nematodes (Suzuki, 2002). Internal symptoms include death of parenchymal cells followed by cavitation and blockage within the tracheids. External symptoms include a reduction in the oleoresin exudation rate followed by the mass propagation of nematodes and wilt of the plant.

In the early stage after infection by nematodes, cytological changes occur in the xylem parenchyma cells causing leakage of cell contents including oily substances which are transported into the surrounding tracheids inducing the dysfunction of the bordered pits in tracheids (Hara & Futai, 2001). The increased production of volatile defence compounds by the plant causes cavitation which disrupts water transportation and finally prevents xylem hydraulic conductance (Kuroda, 1991; Ikeda & Kiyohara, 1995). The secondary resin produced from radial parenchyma cells by nematode infection also may damage the vascular system (Ikeda & Suzaki, 1984).

A reduction in oleoresin exudation rate is the first external symptom of *B. xylophilus* infection due to progressive destruction by the nematodes of epithelial cells in the resin canals (Suzuki & Kiyohara, 1978; Ishida *et al*., 1993). This also marks the onset of advanced stage symptoms. The reduction of oleoresin production can be detected by making a hole of 10-15 mm in diameter through the bark and cambium (Oda, 1967) and this observation was developed as a technique for early diagnosis of pine wilt disease caused by *B. xylophilus* before the nematode was recognised as the causal agent of the disease (Liu *et al*., 1998; Yang *et al.*, 1999).

Cambial death and cavitation in the outer xylem result in a water deficit that reduces transpiration and photosynthesis, causing the first obvious external symptoms of yellowing and wilting of the needles leading to eventual death of the tree (Mamiya, 1983). The wilting may first appear on only one branch ("flag") although the whole tree may later show symptoms (Malek & Appleby, 1984). As a consequence of the reduction of its oleoresin defence mechanism, intensified wilting and yellowing of the needles can be seen from the tree, which becomes attractive to adult insects that gather on the trunk to mate. The tree may die 30-40 days after infection under favourable environmental conditions and may contain millions of nematodes throughout the trunk, branches and roots (Mamiya, 1972).

The physiological water status of the tree and the nematode population density are considered to be the key internal factors in disease development. High temperatures and

drought are external factors that exacerbate the disease. Serious pine wilt disease is associated with higher temperatures and occurs only where the mean summer temperatures exceeded 20ºC (Rutherford *et al*., 1990). Pine wilt disease occurs more frequently and is more destructive in summers with little rainfall (Ge & Xu, 1999).

2.3.2 Factors important in development of pine wilt disease

Pathogenicity of PWN depends on the interaction of the pine species with different PWN populations, the physiological status of the infected pine and environmental conditions (Kiyohara & Bolla, 1990). Differences in nematode populations, numbers of nematode inoculated, life stages of the inoculated nematodes, provenance and health status of the seedlings, environmental conditions and inoculation techniques, may affect the results of pathogenicity tests and these factors have led to great variations among different reports of pathogenicity (McNamara, 2004).

2.3.2.1 Nematode populations

The pathogenicity of *B. xylophilus* associated with conifers varies between different populations. The nematode populations isolated from different coniferous host plants and/or from different regions have been demonstrated as having different virulence on *Pinus* spp. in China (Hu *et al*., 1994; Shen *et al*., 1995; Jiao *et al*., 1996). Pathogenicity differences were also demonstrated in Japan between populations of *B. xylophilus* from *P. thunbergii* and *P. densiflora* in the same and distantly separated pine stands, with virulence ranging from 0-100% (Kiyohara & Bolla, 1990). However, the virulence of nematode populations was found not to vary within a single pine or vector insect (Kiyohara & Bolla, 1990).

The mobility of *B. xylophilus* can also affect the pathogenicity of different nematode isolates (Kawazu *et al*., 1999). The ability of nematodes to move into the cortical tissue has a positive relationship with virulence (Ishida *et al.*, 1993). The movement speed of a pathogenic *B. xylophilus* isolate was higher than that of a non-pathogenic isolate at high population densities (Iwahori & Futai, 1995). However, high mobility has also been found in less virulent North American nematode populations (Rutherford *et al*., 1990) which suggests that the mobility of nematodes is not a major factor in nematode pathogenicity. The migration of the *B. xylophilus* from inoculated branches into the main stem of *P. thunbergii* occurs very rapidly compared to non-host plants (Kuroda & Ito, 1992). A virulent *B. xylophilus* isolate at low temperature (25°C) and avirulent nematodes at high temperature (30°C) could not easily migrate to xylem resin canals or cortical tissue (Ichihara *et al.*, 2000). Recent work has shown

that certain *Pseudomonas* species enhanced reproduction rates of *B. xylophilus* on trees (Zhao & Lin, 2005) and that bacteria associated with the nematode which produce phytoxins also increased egg production and accelerated growth and development of *B. xylophilus* in callus cultures (Zhao *et al.*, 2007).

2.3.2.2 Host plants

Pathogenicity of *B. xylophilus* to different *Pinus* spp. has been investigated in many different studies and the differences in nematode virulence to different *Pinus* species have been demonstrated (Kiyohara & Tokushige, 1971; Mamiya & Kiyohara, 1972; Futai & Furuno, 1979; Dropkin *et al.*, 1981; Dropkin & Linit, 1982; Bedker *et al.*, 1984; Myers, 1984; Ohba *et al*., 1984; Tamura & Dropkin, 1984; Burnes *et al.*, 1985; Dwinell, 1984, 1985; Kaneko & Zinno, 1986; Lee, 1986; Linit & Tamura, 1987; Yang *et al*., 1987; Yang *et al.*, 1988; Kasuya *et al.*, 1990; Schauer-Blume, 1990; Bakke *et al.*, 1991; Sutherland *et al*., 1991; Bai & Cheng, 1993; Xu *et al*., 1994a; Nakamura *et al.*, 1995; Braasch, 1997; Braasch, 2000a; Yang *et al.*, 2002). Latent infections of PWN were also common and varied on different pine species (Halik & Bergdahl, 1994; Bergdahl & Halik, 1999; Yang *et al.*, 2002). Extensive variability was observed in the results obtained from these experiments and this raises the question of whether these results, which used very young *Pinus* spp., related adequately to the natural occurrence of the disease in the field. The inoculation methods for evaluating the pathogenicity of *B. xylophilus* on young *Pinus* plants have been seriously criticized (McNamara, 2004). Seedling tests are particularly vulnerable to variation and the pathogenicity shown on pine seedlings in laboratory tests does not imply any damage on adult trees under natural conditions (Schauer-Blume, 1990).

2.3.2.3 Environmental factors

A close relationship between disease incidence and environmental factors, such as temperature and water content of the soil, has been observed (Mamiya, 1983). Temperature is known to be directly related to pine wilt incidence and inversely related to the disease incubation period. High-temperature stress also predisposes pine trees to lethal infection by *B. xylophilus* (Sikora & Malek, 1991). Inoculation of trees in summer under high temperatures can result in rapid death of trees (as little as 40-60 days), whereas trees inoculated in spring take longer to develop symptoms and inoculations in autumn or winter may result in no development of symptoms (Kiyohara & Tokushige, 1971). Pine seedlings inoculated experimentally and kept at 25-30°C developed symptoms of disease, whereas those

maintained at lower temperatures (15-20°C) did not (Kiyohara, 1973). Pine wilt disease caused by *B. xylophilus* has been recorded mainly in areas where the mean daily summer temperatures are above 20°C (Rutherford *et al*., 1990). Visible symptoms and pine mortality are correlated with the number of nematodes recovered from dead pines, suggesting that temperature may increase the reproduction of nematodes and possibly influenced the host physiology (Melakeberhan *et al*., 1992). Braasch (2000a) also demonstrated that the differences in pathogenic action of *B. xylophilus* and *B. mucronatus* are influenced significantly by temperature.

The conditions of water stress have a positive influence on disease development in pine trees. The failure of water transport after nematode infection and consequently water stress can exacerbate the severity of the symptoms caused by *B. xylophilus* infection (Suzuki & Kiyohara, 1978). The decrease in soil water potential below field capacity accelerated the development of pine wilt disease (Ikeda *et al.*, 1990) and the severity of wilt caused by *B. xylophilus* was affected by water stress (Shimizu *et al.*, 1988). Exposure to acid mist also caused wilting to develop faster in seedlings inoculated with *B. xylophilus* compared to tap water (Futai & Harashima, 1990). The transpiration rate of infected trees falls in the summer months during dry weather and the population of nematodes in the wood increased appreciably. The reduction of transpiration rate was markedly associated with the decrease in the xylem water potential and tended to favour the nematode development, thus the severity of disease development is dependent on the density of the nematode population when transpiration falls (Suzuki, 1984).

Although environmental factors can affect the progression of pine wilt disease and death rate of pine tree, the most important factors in wilting of pine trees by *B. xylophilus* are related to the mechanism of nematode pathogenicity.

2.3.3 Mechanism of pathogenicity

There is still no clear or accurate understanding of the mechanisms underlying the pathogenicity of *B. xylophilus*. A lot of research aimed at elucidating the mechanisms of pathogenicity has been carried out especially in Japan and China. In general, there are conflicting views as to whether pine wilt disease is caused solely by the nematode or by a complex consisting of the nematodes and associated bacteria.

2.3.3.1 Causes of pine wilt disease

2.3.3.1.1 Nematode as the sole pathogen

Bursaphelenchus xylophilus was demonstrated as the causal agent of pine wilt disease when inoculation of *Pinus* spp. with pure nematode suspension (cultured on fungi) killed healthy pine trees whereas fungus alone or filtrate from the nematode/fungus culture did not (Kiyohara & Tokushige, 1971). Mamiya (1972) confirmed these results by inoculating healthy 7-year-old *P. thunbergii* and 15-year-old *P. densiflora* with a water-suspension of the nematodes, resulting in death of the tree. Bolla and Jordan (1982) demonstrated that axenically cultured *B. xylophilus* sterilized with streptomycin sulphate, Nystatin and penicillin-G caused complete wilting of *P. sylvestris* seedlings within 3-5 days of infection. Kuroda and Ito (1992) also demonstrated that *B. xylophilus* was the causal agent of pine wilt rather than any succeeding bacterial or fungal pathogens through nematode infection of *P. thunbergii.*

2.3.3.1.2 Nematode and bacterial disease complex

A potential role for bacteria associated with *B. xylophilus* in the disease process has been proposed. A series of studies has shown that bacteria from various genera including *Bacillus* (Kawazu *et al.*, 1998), *Pseudomonas* (Oku *et al*., 1980; Hong *et al*., 2002; Han *et al*., 2003), *Xanthomonas* (Higgins *et al.*, 1999b) and *Pantoea* (Hong *et al*., 2002; Han *et al*., 2003) are associated with *B. xylophilus*. Structural studies demonstrated that no bacteria were present within the body of *B. xylophilus* but the presence of bacteria on the nematode surface was described (Kusonoki, 1987; Zhao *et al*., 2000b; Xie *et al*., 2002). Large numbers of bacteria have been described as being associated with damaged areas of plants infected with *B. xylophilus* (Kusonoki, 1987; Zhao *et al.*, 2000b). In addition, it has been shown that *Pseudomonas* and *Pantoea* were present in trees infected with *B. xylophilus* but were absent from uninfected trees (Hong *et al.*, 2002; Han *et al*., 2003). These studies also provided perhaps the most convincing evidence for bacteria carried by nematodes playing a role in pathogenicity during the wilting process. It was suggested that pine wilt disease is caused by co-infection of both *B. xylophilus* and bacteria and possibly involved toxic effects of bacteria (Han *et al.*, 2003; Hong *et al.*, 2003).

2.3.3.2 Pathogenesis for pine wilt disease

An interest in understanding the processes that lead to pine wilting so rapidly after *B. xylophilus* infection, has stimulated a vast amount of research on mechanisms of pathogenicity. It has been suggested that pine wilt may be related to enzymes, phytotoxins, chemicals or toxins produced by nematodes, trees or bacteria associated with the nematodes.

2.3.3.2.1 Enzymes

Enzymes, especially cellulase, produced by *B. xylophilus,* could destroy host cell walls and cell membranes causing oleoresin to leak and diffuse into tracheids, blocking water conductance and finally causing pine wilt (Yamamoto *et al.,* 1986). Cellulase activity was detected in crude *B. xylophilus* extracts (Odani *et al.*, 1985a; Jiang & Wang, 1995; Yan & Yang, 1997) and at least 5 cellulase isozymes have been shown to be produced by nematodes (Odani *et al.*, 1985b) while they migrated. Pine shoots show symptoms of necrosis when treated with cellulase (Yamamoto *et al*., 1986), and it has been suggested that the dissolution patterns appearing in cell walls are caused by the cellulase activity of the nematode (Kusunoki, 1987). Kojima *et al.* (1994) demonstrated that the virulent and less virulent *B. xylophilus* isolates all produced essentially similar cellulases which were different from those of *B. mucronatus.* The cellulase (endo-β-1,4-glucanase) genes have been cloned and functionally characterized in *B. xylophilus* (Kikuchi *et al.*, 2004). It has also been suggested that cellulase secreted by the pine wood nematode was responsible for pathogenicity in pine wood nematodes (Kojima *et al.*, 1994). These enzymes are secreted by both virulent and less virulent *B. xylophilus* and also *B. mucronatus* isolates, suggesting that although they help the movement of nematode through the plant cells their role in pathogenicity remains unclear (Kojima *et al.*, 1994).

Other enzymes were also found to be involved in the disease progress. Yan and Yang (1997) showed that protease, peroxidase and amylase are present in *B. xylophilus* secretions and suggested that all of these enzymes may play a role in the primary reaction of the disease after nematode infection. The production of chitinase by *Bursaphelenchus* spp. could play a role in pathogenicity of the nematode and it has been suggested that variation in chitinase is correlated with variations in the pathogenicity of pine wood nematode isolates (Higgins *et al.*, 1999a).

2.3.3.2.2 Chemicals

It has been suggested that terpenes are important in development of pine wilt disease. The concentration of volatile terpenes was increased in xylem tissue after infection by *B. xylophilus*. High concentrations of terpenes are also present in tracheids and may cause cavitations in the tracheids that interrupt water conduction in sapwood cause death of the tree (Kuroda, 1989; Kuroda *et al.*, 1991). Further evidence for the role of terpenes in disease was obtained by direct injection of alpha-pinene into healthy pine trunks which led to formation of artificial cavitation in xylem (Kuroda, 1991). Modified monoterpenes with aldehyde and ketone carbonyl functional groups were detected in *P. sylvestris* shortly after *B. xylophilus* infection (Bolla *et al.*, 1984). Increasing release of ethylene from *P. thunbergii* seedlings was also detected after inoculation with virulent isolates of *B. xylophilus* and it was suggested that this coincided with cambial death in seedlings infected with the virulent nematodes (Fukuda *et al.*, 1994).

The relationships between terpenoid content and resistance of *P. massoniana* to *B. xylophilus* were studied (Zhao *et al.*, 1999). The presence of some terpenoids (e.g. longifolene, cedrene, farnesene, alpha-terpineol, sandaracopimadiene) was positively correlated with the resistance of the tree. However, the differences in the terpenoid types and their levels in trees before and after nematode infection were unclear. Difficulties in understanding mechanisms underlying the production and role of terpenoids has prevented further research in this area.

2.3.3.2.3 Phytotoxins

The rapid death of *Pinus* spp. after *B. xylophilus* infection suggests the involvement of phytotoxins in pine wilt disease. These phytotoxins maybe produced by the pine tree after nematode infection, by the nematode itself or by symbiotic bacteria carried by nematodes.

2.3.3.2.3.1 Production of phytotoxins by infected plants

The observation that cell death in the host occurred in advance of the increase of nematode population led to speculation that the nematode may metabolize some component of the pine into the toxin (Oku *et al.*, 1979). Phytotoxins isolated from nematode infected plants were subsequently identified and characterised (Bolla *et al*., 1982). It was suggested that these toxins are synthesized shortly after nematode infection (Bolla *et al.*, 1984) and suppressed water transport in the trees (Shaheen *et al.*, 1984).

Some phytotoxins only occurred in susceptible seedlings after nematode infection (Bolla *et al*., 1986), including benzoic acid (BA), catechol, dihydroconiferyl alcohol, 8-hydroxycarvotanacetone and 10-hydroxyverbenone (Oku, 1984). The toxicity of some of these metabolites correlated positively with the susceptibility of pines to *B. xylophilus*. Some of these metabolites showed synergistic toxicity when present in combination. The D-isomer of 8-hydroxycarvotanacetone, dihydroconiferyl alcohol and 10-hydroxyverbenone inhibited the reproduction of *B. xylophilus* and were prominently toxic to susceptible pine species (Oku, 1988). Toxic substances that induced wilting were also detected by Cao *et al.* (2001) in artificially or naturally-infested pine trees. These included benzene acetic acid and 2-methoxycinnamic acid and these compounds were considered to be the most important secondary metabolites synthesized in nematode infested pines (Cao *et al.*, 2001). BA was found in nematode-infected plants (Bolla *et al.*, 1982) and the toxicity of this compound to plants was confirmed using different tests (Ikeda *et al.*, 1989; Mamiya *et al.*, 1989). The formation and accumulation of BA and its conjugates in plants has also been induced by phenylacetic acid (PA) which may be produced by associated bacteria of nematodes (Oku, 1990; Kawazu *et al.*, 1996a; Zhang *et al.*, 1997).

2.3.3.2.3.2 Production of phytotoxins by bacteria associated with nematodes

When filtered culture medium used to grow bacteria associated with nematodes is applied to callus material derived from trees, cell death in the callus is observed (Han *et al*., 2003). This suggests that bacteria produce a phytotoxin. Nematodes cultured axenically did not produce phytotoxin (Cao *et al.*, 2001) and were not as pathogenic as *B. xylophilus* extracted from naturally infected trees (Kawazu & Kaneko, 1997; Cao *et al.*, 2001). Zhao *et al*. (2003) identified 24 bacterial strains on *B. xylophilus* collected from diseased trees and 17 of these produced phytotoxins. They suggest that pine wilt disease is a complex, induced by both PWN and the bacteria it carries.

Another study suggested that the wilt toxin emanating from *B. xylophilus* is a metabolite of *Pseudomonas* sp. which was capable of infecting the whole tree (Oku *et al*., 1980). Tada *et al.* (1981) also confirmed that a bacterium isolated from pine wood nematode had the ability to produce toxin. Bacteria carried by *B. xylophilus* produced toxic PA which was suggested as the causal factor in disease development in *P. thunbergii* (Mori & Inoue, 1986; Oku, 1990; Kawazu, 1996b; 1997), and the reactions of pine tissues to the PA toxin may be important in the pathogenesis mechanism for the pine wilt disease (Kawazu *et al.*, 1996b). No wilt symptoms appeared on aseptic pine seedlings after inoculation with the aseptic nematodes

under aseptic conditions, further supporting a role for bacteria in pine wilt disease (Kawazu & Kaneko, 1997). Seedlings inoculated with *B. xylophilus* isolate which carried PA-producing bacteria wilted, while those inoculated with nematode alone did not wilt (Kawazu *et al.*, 1999; Zhao *et al*., 2000c). PA was considered as the pathogenic toxin and the PA-producing bacterial strains accompanying the pathogenic nematode was considered as the genuine pathogens of the pine wilt disease (Zhang *et al.*, 1997).

Although the studies outlined above apparently provide convincing evidence for the role of bacteria involved in the pathogenicity of *B. xylophilus* to *Pinus* spp., several notes of caution need to be considered. Bacteria alone cannot cause disease (Han *et al.*, 2003), and in addition, using callus or very young seedlings as host material in tests for assessing pathogenicity can allow *B. mucronatus* scored as highly pathogenic. Data on pathogenicity mechanisms obtained from tests using aseptic callus and young seedlings are suspect because this situation would never exist in the field (Yang, 2002).

It is possible that *B. xylophilus* is the causal agent for pine wilt disease and phytotoxins produced by the associated bacteria may bring about earlier death of a host.

2.3.4 Pathogenicity-related nematode genes

The major sedentary endoparasitic nematodes, root-knot nematodes and cyst nematodes, can modify one or more plant cells into a specialized feeding site that supports the development of the nematode to the adult stage. Because of the huge economic losses attributable to these nematodes a great deal of research has been performed aiming to understand the molecular basis of the host-parasite interaction. This has led to the identification of a variety of genes encoding proteins important in the host parasite interaction.

2.3.4.1 Sedentary endoparasites

Plant-cell-wall-degrading enzymes produced by a variety of plant-parasitic cyst and root-knot nematodes, including *Heterodera*, *Globodera* and *Meloidogyne* species, have been extensively studied. Several genes encoding cell-wall-degrading enzymes, such as cellulases (β-1,4-endoglucanases) (Ding *et al*., 1998; Smant *et al*., 1998; Rosso *et al.*, 1999; Goellner *et al.*, 2001; Gao *et al*., 2002), pectate lyases (de Boer *et al*., 2002; Doyle & Lambert, 2002; Huang *et al.*, 2005; Popeijus *et al*., 2000), xylanase (Dautova *et al*., 2001), polygalacturonase (Jaubert *et al*., 2002) and expansin (Qin *et al.*, 2004) have been identified in these nematodes. These enzymes are produced within the subventral oesophageal gland cells of the nematodes

and are secreted through the nematode stylet during invasion of plant tissues and are thought to facilitate penetration and migration of nematodes to their feeding sites (de Boer *et al*., 1999). These cell-wall-degrading enzymes are endogenous and not found in other nematodes or almost any other animals, and are most similar to bacterial genes. This has led to the suggestion that they have been acquired *via* horizontal gene transfer (HGT) from bacteria (Yan *et al.*, 1998; Jaubert *et al*., 2002; Jones *et al*., 2003; 2005; Scholl *et al.*, 2003).

2.3.4.2 Migratory endoparasites

Until recently little was known about the molecular basis of host–parasite interactions in *Bursaphelenchus* species. An expressed sequence tag (EST) project on *B. xylophilus* (Kikuchi *et al*., 2007) allowed the cloning and functional characterization of cellulase (endo-β-1,4-glucanase) genes from *B. xylophilus*. It was shown that, as in sedentary endoparasites, the *B. xylophilus* cellulases are secreted through the stylet and may soften the plant cell wall to facilitate their feeding and migration. The *B. xylophilus* cellulases showed most similarity with fungal cellulases and were classified into glycosyl hydrolase family (GHF) 45 which suggested that these genes were acquired by horizontal gene transfer (HGT) from fungi on which ancestors of the current nematode species fed (Kikuchi *et al.*, 2004). By contrast, the cellulases of cyst and root-knot nematodes belong to GHF5 and are most similar to bacterial cellulases. It was therefore proposed that cyst or root-knot nematodes and *Bursaphelenchus* spp. have evolved both the ability to digest cellulose and the ability to parasitize plants independently (Kikuchi *et al*., 2004), with the horizontal transfer of genes encoding cell wall degrading enzymes having played a key role in evolution of plant parasitism by nematodes on each occasion. In addition, genes encoding pectate lyase were also identified in ESTs from *B. xylophilus* and *B. mucronatus*. Two pectate lyase genes (*Bx-pel-1* and *Bx-pel-2*) were cloned and characterized from *B. xylophilus*. The deduced amino acid sequences of these pectate lyases are most similar to polysaccharide lyase family 3 proteins. The genes are expressed in the oesophageal gland cells of the nematode indicating that, like the cellulases, the pectate lyases could be secreted into plant tissues to help feeding and migration in the tree. This suggests that pectate lyases are widely distributed in plant-parasitic nematodes and may play an important role in plant–nematode interactions (Kikuchi *et al*., 2006).

More recently, ESTs that may encode expansin-like proteins have been identified in the EST dataset from *B. xylophilus* and *B. mucronatus*. Similar proteins are present in cyst nematodes (Qin *et al*., 2004) and are thought to break non-covalent bonds between cellulose

microfibrils in plant cell walls. No work has been performed on these genes in *B. xylophilus* or *B. mucronatus*.

In addition to enzymes that modify the plant cell wall, endo-β-1,3-glucanases, which catalyse the hydrolysis of β-1,3-D-glucosidic linkages in β-1,3-D-glucan, have been characterized from *B. xylophilus* and *B. mucronatus* (Kikuchi *et al*., 2005). These enzymes are widely distributed among bacteria, fungi and higher plants but are not usually present in animals. In the animal kingdom, functionally characterized endo-β-1,3-glucanases are restricted to marine invertebrates, insects and other invertebrates. Since endo-β-1,3-glucan is the main structural component of fungal cell walls and fungi are the main food source for *Bursaphelenchus* spp., it seems likely that endo-β-1,3-glucanases play an important role in the feeding process of the nematodes. The *B. xylophilus* endo-β-1,3-glucanase gene is expressed in the oesophageal gland cells and the protein is secreted from the nematode stylet. The deduced amino acid sequence of the gene is very similar to glycosyl hydrolase family 16 proteins. Sequence comparisons suggest that the gene encoding the endo-β-1,3-glucanase was acquired by HGT from bacteria. Therefore *B. xylophilus* contains genes that have been acquired by HGT from both bacteria and fungi, which supports the idea that multiple independent horizontal gene transfer events have helped in shaping the evolution of several different life strategies in nematodes (Jones *et al*., 2005).

Understanding the interaction between plant-parasitic nematodes and their host plants is essential for designing disease control programs that are based on the structure and function of secreted proteins encoded by nematode parasitism genes. These proteins secreted from gland cells of nematodes maybe recognised by the plant cells during the host-nematode interaction and trigger host defence reactions including the hypersensitive response (HR). Sequence variations in these secreted proteins may reveal the changes in amino acids which may correlate with virulence phenotypes. For example, the *Avr3a* gene from the oomycete *Phytophthora infestans* was identified through screening ESTs for genes encoding secreted proteins, followed by association genetic studies (Armstrong *et al*., 2005). The protein encoded by *Avr3a* is recognised in the host cytoplasm where it triggers *R3a*-dependent cell death in an avirulent interaction. Small changes in the amino acid sequence of the AVR3A protein lead to a failure of the host to recognize the protein and a compatible reaction. No virulence associated proteins have been identified in nematodes to date.

2.3.4.3 RNAi for gene function analysis

Different molecular techniques have been used to identify nematode parasitism genes that encode secreted proteins from different plant-parasitic nematodes. Novel strategies for interfering with these pathogenicity factors are currently being developed (Davis *et al*., 2000).

Several strategies have been investigated to genetically transform plant-parasitic nematodes but none of these approaches has been reliably successful (Davis *et al.*, 2000). An alternative strategy is the use of RNA interference (RNAi) technology as an investigative tool for target genes that might also provide a further basis for transgenic resistance.

RNA silencing triggered by double-stranded RNA (dsRNA) was first demonstrated for *Caenorhabditis elegans* by Fire *et al.* (1998). Since that time it has been used with different organisms including protozoa, animal parasitic nematodes, mammals, plants and insects. RNAi provides an experimental tool to help determine gene function in many organisms and may also offer new approaches to pathogen control (Bakhetia *et al*., 2005). This technique is based on the innate defence mechanisms of organisms against virus infection that recognize dsRNA. Gene silencing uses RNA polymers synthesized *in vitro* to produce dsRNA homologous to the target gene, which are then introduced into the organism. This can lead to digestion of the dsRNA into small fragments which then leads to the post-transcriptional breakdown of the target mRNA. The RNAi phenotype can be inherited for at least two generations (Grishok *et al*., 2001) depending on the life cycle time.

Applying RNAi to plant-parasitic nematodes presents particular problems. Their small size makes microinjection with dsRNA a major technical challenge. The obligate parasitism and relatively long life cycles are additional limiting factors on the rate of progress of such studies. The sedentary obligate parasites do not normally feed until they have infected a host plant and they spend most of their life cycle inside the host's roots. The only readily available life stage that one can easily obtain and handle are the eggs and second-stage juveniles (J2s). The J2s are the infective stage and both this stage and eggs are non-feeding. The uptake of dsRNA by the pre-parasitic J2s has been achieved in two cyst nematodes, *Heterodera glycines* and *Globodera pallida,* using octopamine to stimulate the nematode to take up the dsRNA (Urwin *et al*., 2002). This technique was used to study silencing of a cysteine proteinase in *H. glycines* and *G. pallida,* the C-type lectin (*hgctl*) in *H. glycines* and the major sperm protein (*msp)* in *G. pallida*. These three genes were chosen to evaluate the usefulness of the technique in relation to different aspects of the parasites' biology. A reduction in the

expression levels of the cysteine proteinase and *hgctl* was demonstrated in the treated J2s and following silencing of the *msp* gene there was a reduction in the transcript level of this gene in the dsRNA treated nematode 15 days after treatment as assessed by Northern blot analysis. Two genes from *G. rostochiensis* were analysed by Chen *et al*. (2005) using RNAi, the subventral gland expressed protein β-1,4-endoglucanases and the amphid protein Gr-AMS. The dsRNA for endoglucanase was generated from a homologous region with very high similarity to the *Gr-eng-1* and *Gr-eng-2* genes as well as a high similarity to *Gr-eng-3,4* sequences. The phenotypic effect of silencing β-1,4-endoglucanase was to reduce the ability of the nematode to infect the plant, whereas silencing the *Gr-ams* gene showed a reduced ability of the treated nematodes to locate the host root. In another study, the RNAi technique was used by Bakhetia *et al.* (2005) to study the function of the *Meloidogyne incognita* dual oxidase gene. The phenotypic effect of silencing dual oxidase was to reduce the number of nematodes developing in the plant and also a reduction in the rate of development of the nematode. Reduction in the transcription level of this gene in the dsRNA treated nematodes was also demonstrated using RT-PCR and real time PCR. Another method for inducing the uptake of dsRNA by J2 of *M. incognita* was described by Rosso *et al.* (2005) whereby nematodes were incubated in 1% resorcinol for 4h. This method was used in studies of the subventral gland expressed genes calreticulin and polygalacturonase and showed reduced infection rates after treatment with dsRNA. In addition, it was reported that transcription was reduced only for a limited time, up to 68h after soaking. Eggs of root-knot nematodes have been targeted for RNAi by Fanelli *et al*. (2005) who silenced the chitin synthase gene, resulting in arrested development of the eggs.

Nematode FMRFamide-like peptides, which modulate sensory and motor functions, were analysed by RNAi in *G. pallida* (Kimber *et al.*, 2006). Silencing any of the five characterized *flp* genes (*Gp-flp-1*, *-6*, *-12*, *-14,* or *-18*) of *G. pallida* gave rise to distinct aberrant behavioural phenotypes consistent with key roles in motor function.

RNAi has also been used to identify essential genes of plant-parasitic nematodes that may serve as novel control targets. An EST database comparison between *H. glycines* and *C. elegans* yielded a total of nearly 8334 conserved genes, 1508 of which have lethal phenotypes/phenocopies in *C. elegans (*Alkharouf *et al*., 2007). RNAi of a conserved ribosomal gene from *H. glycines* (*Hg-rps-23*) gave a lethal phenotype.

No RNAi studies have been reported for any migratory parasitic nematodes.

Chapter 3

General materials and methods

In this chapter only those methods and materials are discussed that are used in more than one chapter. Specific materials and methods are described in the appropriate chapters.

3.1 Nematode extraction and culture

3.1.1 Extraction

Samples were collected from dead pine trees or wooden packaging materials and consisted of about 10-cm-long pieces, which were subsequently cut into smaller pieces of no more than 1 cm. Nematodes were extracted using the modified Baermann funnel technique (Southey, 1986) for 48 h at ±25°C. The technique was also used for the extraction of nematodes cultured on *Botrytis cinerea*.

3.1.2 Culturing

Extracted nematodes were picked up with a fine needle from the nematode suspension and morphological characters were observed under a dissecting microscope. Nematodes belonging to the genus *Bursaphelenchus* were transferred into sterilized distilled water (SDW) drops and washed three times. Eventually, for each isolate, 10-20 nematodes with similar morphological characters were inoculated on a thick layer of non-sporulating *B. cinerea* mycelium, which was grown on potato dextrose agar (PDA) in a 10 cm diam Petri dish.

3.1.3 Purification

After incubation at ±25ºC for 2 weeks, the nematodes were picked up from the culture and observed under microscope to ensure that they were without any contamination. Gravid females were selected and inoculated one by one separately into a small drop of SDW on a *B. cinerea* culture and incubated at ±25ºC for 2-3 weeks. Five replicates were prepared for each isolate. Eventually, one of the successful replicates was selected and the nematodes were transferred onto fresh *B. cinerea*.

3.1.4 Maintenance

All purified nematode isolates were kept on *B. cinerea* dishes at ±25ºC for 7-10 days. When the fungus culture disappeared and a massive number of nematodes was observed in the dishes, the dishes were transferred at 4ºC for preservation. All isolates were recultured at 3-month intervals.

3.2 Agarose gel electrophoresis

Following DNA amplification, 5μl of each PCR product was mixed with 1μl of 6 × loading dye (Promega, Leiden, The Netherlands) and loaded on a 1% agarose gel stained with 0.003% ethidium bromide (0.02 μg/ml) in 1 × TAE buffer (Sambrook *et al.*, 1989). A 100 bp DNA marker (Promega, Leiden, The Netherlands) in 5μl volume was loaded on the gel as size marker. After electrophoresis for 1 hour at 100V, the DNA bands were visualized on an UV-transiluminator and photographed. The PCR products were kept at -20°C or used immediately.

3.3 PCR product purification from agarose gel

Prior to the start of the purification process, a water bath was set at 50°C and 1.5 ml Eppendorf tubes were weighed. Then the complete PCR product was loaded on a 1% agarose gel with large wells; electrophoresis was run for one hour and DNA fragments were excised from the agarose gel with a clean sharp scalpel under UV light. Further purification of DNA was executed using the MinElute gel extraction kit (Qiagen) following the manufacturer's instructions.

Briefly, the weight of the agarose was determined and 3 volumes of QG buffer were added. The gel and liquid were then heated at 50°C for 10 min with vortexing every 3 min. A volume of isopropanol, equal to the volume of the original gel piece, was then added to the sample and mixed. The mixture was transferred to a MinElute column in a 2ml collection tube and centrifuged for 1 min. The flow-through was discarded and the column was washed with 500μl QG buffer and centrifuged for 1 min. The flow-through was discarded and the column washed by adding 750μl PE buffer and centrifuged twice for 1 min, discarding the flow-through each time in order to dry the column.

The DNA was eluted by placing the column in a new 2ml tube and adding elution buffer (EB) from 10 to 50μl to the centre of membrane in tube. The EB volume was adjusted depending on the expected DNA concentrations. After one minute incubation at room temperature the tube was centrifuged for 1 min. The purified PCR product was finally collected and stored at -20°C for future use. All centrifugation steps were at 13,000 × *g*.

3.4 Cloning PCR products

Purified DNA was ligated into pGEM-T Easy vector (Promega) in the presence of 1× ligation buffer and T4 DNA ligase according to the manufacturer's instructions. Ligation reactions were incubated for 1 hour at room temperature or overnight at 4°C. Two microliters of ligation reaction were transformed into DH5α competent cells (Invitrogen) by electorporation using a MicroPulser (BIO-RAD) or transformed into *Escherichia coli* strain JM109 using a heat shock protocol (Promega).

The transformed cells were incubated for 1h at 37°C in 900μl SOC media then spread on Luria Broth (LB)-plates (Trypton peptone 10% w/v, Yeast extract 5% w/v, NaCl, 5%w/v) containing ampicillin (100 μg/ml), IPTG and X-Gal (LBAIX) and incubated overnight at 37°C. White colonies were selected and each one was picked off and resuspended in 20μl of SDW, 10μl were plated on a fresh LBAIX plate as a reference and 5μl were used as template in colony PCR with vector primer pair (M13F: 5'-CAG GAA ACA GCT ATG AC-3' and M13R: 5'-GTT TTC CCA GTC ACG AC-3'). PCR products were visualized after electrophoresis as described above. The colonies that had the expected size of inserts were selected. These were re-cultured in 2-5 ml LB with 100μl /ml ampicillin shaking (150 *rpm*) overnight at 37°C. The contents were used for further plasmid DNA extraction or stored (-20°C) for later use.

3.5 Plasmid DNA isolation

Plasmid DNA was extracted from overnight cultures using the QIAprep spin Miniprep kit (Qiagen). The overnight culture was centrifuged at 3000 × *g* for 5 min, the supernatant was discarded and the bacterial pellet was resuspended in 250μl buffer P1. Buffer P2 (250μl) was added and the tube was inverted gently 6 times. Subsequently, 350μl of N3 buffer was added and the tube was gently inverted 6 times followed by centrifugation at 13,000 × *g* for 10 min. The resulting supernatant was transferred into a QIAprep Spin Column placed into a 2ml collection centrifuge tube. This tube was centrifuged at 13,000 × *g* for 1 min and then the column was washed by adding 0.5ml of PB buffer and centrifuging again at 13,000 × *g* for 1 min. The column was then washed with 0.75ml PE buffer and centrifuged for 1 min as above.

After each centrifugation step, the flow-through was discarded and a final centrifugation for 1 min was performed to dry the column. Finally the DNA was eluted by placing the column into a new centrifuge tube and adding 50μl of EB buffer (10mM Tris-Cl, pH 8.5) to the centre of the column. After 1 min at room temperature this was then centrifuged for 1 min

and the flow through was collected. The purified plasmid DNAs were kept at -20°C or subsequently used for sequencing.

3.6 Sequencing

Sequencing reactions were carried out using an ABI Prism® BigDye TM terminator cycle sequencing kit (AB Applied Biosystems). The cycle sequencing reaction was carried out in a total volume of 10μl and contained 300ng of the plasmid, 1μl of 10 μM primer (M13F or M13R), 2μl 5 × sequencing buffer and 2μl big dye reaction mix. The PCR conditions were 94°C 2 min followed by 25 cycles of 94°C 10 sec, 50°C 5 sec and 60°C 4 min. Sequencing products were cleaned by ethanol-salt precipitation. For this 1/10 volume sodium acetate, 1/10 volume 125mM EDTA and 2.5 volumes absolute ethanol were added to each reaction. The reaction was left at room temperature for 15 min to precipitate the extension products. After centrifuging at 13,000 × *g* for 20 min, the ethanol was removed carefully and the pellet was washed with 70% ethanol and centrifuged for 5 min at 13,000 × *g*. The ethanol was discarded and pellet was dried at 50°C for 5-10 min or 90°C for 1 min. The sequence reactions were run on an Applied Biosystems ABI 377 or 3730 DNA sequencer.

To be able to sequence the complete cloned PCR product, both DNA strands were sequenced from all samples by using vector primers (see above) during the cycle sequencing reaction. With the help of the software packages Chromas Lite (version 2.0) and BioEdit Sequence Alignment Editor (Hall, 1999), the full sequence was generated. All sequences were aligned using the program ClustalW version 1.8 (Thompson *et al*., 1997) with default options. Phylogenetic analysis was performed using PAUP 4.0b10 (Swofford, 2002) or MEGA 3.0 (Kumar *et al*., 2004).

Chapter 4

Morphological, morphometrical and molecular characterization of **Bursaphelenchus** *spp. isolated from imported packaging wood in Nanjing, China*[*]

[*]Adapted from**: Li, H.,** Trinh, P. Q., Waeyenberge, L. & Moens, M. (2008). *Bursaphelenchus chengi* sp. n. (Nematoda: Parasitaphelenchidae) isolated at Nanjing, China, in packaging wood from Taiwan. *Nematology* 10, 335-346.

Li, H., Trinh, P. Q., Waeyenberge, L. & Moens, M. (2008). Morphological, morphometrical and molecular characterization of *Bursaphelenchus* spp. isolated from imported packaging wood in Nanjing, China. *Nematology*, submitted.

4.1 Introduction

As international trade is booming in China, the threat of introducing non-indigenous nematode diseases via commodities and their packaging is increasing. Most of the shipping containers are produced from unprocessed lower quality hardwood or coniferous wood. These woods are more likely to harbour pests such as PWN and its vector species of the genus *Monochamus* (Braasch *et al.*, 2004a). Wood packaging material untreated by chemicals or heat may also contain other *Bursaphelenchus* species (Evans *et al*., 1996). It has frequently been proven that PWN can be spread over long distances via the wood used for the packing of shipped commodities (La *et al.*, 1999; Suzuki, 2004; Yang, 2004). The border or post-border inspections of packaging wood or dead pine trees are considered the most effective measure to prevent the introduction and distribution of PWN (Braasch *et al.*, 2004a). Despite the strict phytosanitary measures that have been implemented by the EU, *B. xylophilus* has been detected in 1998 in Portugal for the first time in Europe (Mota *et al.*, 1999). Based on genetic data it was suggested that the PWN was introduced to Portugal from imported packaging wood of East Asian origin (Vieira *et al.*, 2007).

Since the pine wilt disease was first discovered in China in 1982 in the purple mountains of Nanjing (Cheng *et al*., 1983), the damage caused by *B. xylophilus* is still increasing and the infected area has expanded to parts of the provinces of Jiangsu, Anhui, Guangdong, Shandong, Zhejiang and Hubei (Yang, 2004). It is thought that *B. xylophilus* originally entered China by means of wooden packaging materials from an infested country (Yang, 2004). Currently, almost all imported packaging wood is inspected by the Chinese Entry-Exit Inspection and Quarantine Bureaus.

Whilst participating in the inspection of wood packaging materials imported at Nanjing Port in 2003-2004, I detected several *Bursaphelenchus* isolates in samples from wood packaging commodities from eight countries and regions; one of isolates proved to be an undescribed species of *Bursaphelenchus.* In this chapter, the variation in morphology, morphometrics and rDNA-ITS region of the described *Bursaphelenchus* isolates are reported with the comparison of isolates of three species which were earlier detected in China. The new species is described as *Bursaphelenchus chengi* in memory of Dr Hurui Cheng who was the first to report the pine wilt disease in China.

4.2 Materials and methods

4.2.1 Nematode isolation and culture

Thirteen isolates (Table 4.1) were collected from fragments taken from packaging wood which were cut into smaller pieces of no more than 1 cm wide. Nematodes were extracted and cultured on *B. cinerea* (see chapter 3). Three *Bursaphelenchus* isolates of Chinese origin and kept in a live collection at Nanjing Agricultural University (Table 4.1) were used for comparisons.

Table 4.1 Origin of isolates and Genbank accession number for D2D3 rDNA sequences for *Bursaphelenchus* species isolated from imported wood packaging at Nanjing, China.

Isolate	Code	Origin	Species	Genbank accession number
Wood packaging	BxJAP	Japan	*B. xylophilus* R-form	EU295504
	BxCAN	Canada	*B. xylophilus* M-form	EU295500
	BmCAN	Canada	*B. mucronatus* East-Asian type	EU295503
	BmHK	Hongkong	*B. mucronatus* East-Asian type	EU295493
	BmGER	Germany	*B. mucronatus* European type	EU295492
	BmMEX	Mexico	*B. mucronatus* European type	EU295494
	BmSWD	Sweden	*B. mucronatus* European type	EU295495
	BdKOR	South Korea	*B. doui*	EU295499
	BdJAP	Japan	*B. doui*	EU295501
	BrGER	Germany	*B. rainulfi*	EU295496
	BrKOR	South Korea	*B. rainulfi*	EU295498
	BtHK	Hongkong	*B. thailandae*	EU295497
	Bursaphelenchus sp. n.	Taiwan	*B. chengi*	EU107359
Comparison	BxLYG	Lianyungang, China	*B. xylophilus*	EU295491
	BmNJ	Nanjing, China	*B. mucronatus* East-Asian type	EU295502
	BdZJ	Zhejiang, China	*B. doui*	EU295505

4.2.2 Morphology and morphometric observations

For morphological observations, nematodes collected from the *Botrytis* cultures were mounted in water and heat-relaxed on temporary slides, observed under an Olympus BX-51 light microscope, and photographed with an Olympus U-TV 0.5xC-3 digital camera.

Measurements were taken from nematodes mounted in permanent slides after being heat-killed with fixative F.A. (4:1) and dehydrated with ethanol-glycerin according to Seinhorst (1959) modified by De Grisse (1969). The morphometrics of all isolates along with three comparable isolates from China were taken according Ryss *et al.* (2005) who emphasized the importance of spicule characters of the male. Extra morphometrical data were added, especially that on spicule characters of the males.

For described *Bursaphelenchus* species, the morphometrics of the five identified species were compared with the original descriptions and related references. Morphometrical data of males and females of all isolated *Bursaphelenchu*s species were analysed using a forward stepwise Canonical Discriminant Analysis (CDA) (Genstat 10.0).

For the newly described species *B. chengi,* the specimens were prepared for scanning electron microscope (SEM) observations as described by Eisenback (1985). The characters were viewed and photographed using a JEOL 35 scanning electron microscope. The spicules were excised and prepared for SEM observations by the method described by Peñas *et al.* (2006).

4.2.3 Molecular characterisation

4.2.3.1 DNA extraction

The procedure used for DNA extraction was as described in He *et al*. (2003) with some modification as described in Li *et al.* (2008). Ten to 20 nematodes were transferred into 30μl 2 × worm lysis buffer (WLB) (20 mM Tris-HCl pH 8.0, 100 mM KCl, 3.0 mM Mg_2Cl, 2.0 mM DTT, 0.9% Tween 20) and cut into two to three fragments with a sterilised scalpel. Ten μl WLB along with all nematode fragments were pipetted into 8 μl ddH_2O with 2 μl proteinase K (60 μg/ml) in an Eppendorf tube, which was then briefly spun and stored at -70°C for at least 10 min. Subsequently, the Eppendorf tube was incubated at 65 °C for 1-2 hr and the proteinase K was denatured at 95°C for 10 min. Finally, the DNA suspension was cooled to 4°C and stored at -20°C for further applications.

4.2.3.2 ITS-RFLP profiles

The ITS-RFLP analysis was carried out as described by Braasch and Burgermeister (2002). A fragment of nematode rDNA containing the internal transcribed spacer regions ITS1 and ITS2 was amplified by PCR using the forward primer 5'-CGT AAC AAG GTA GCT GTA G-3' (Ferris *et al*., 1993) and the reverse primer 5'-TTT CAC TCG CCG TTA CTA

AGG-3' (Vrain, 1993). The PCR mixture (50 μl) contained 0.6 μM of each primer, 2 units *Taq* DNA polymerase (Promega, Leiden, The Netherlands), 10 mM Tris-HCl pH 8.8, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM dNTP's and 2 ng DNA template. Amplification was carried out using a PTC-100/200 thermocycler (MJ Research, San Diego, CA, USA) employing an initial denaturation step at 94°C for 2.5 min, 40 reaction cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension step at 72°C for 5 min. After PCR, 5 μl of amplified product was visualized by electrophoresis in a 1% agarose gel under UV light (see chapter 3).

Eight microlitres of the amplified DNA was digested (Braasch & Burgermeister, 2002) for at least 4 h at 37°C using 10U of one of the following restriction enzymes, *Rsa*I, *Hae*III, *Msp*I, *Hinf*I and *Alu*I, following the manufacturer's instructions (Promega, Leiden, The Netherlands). Fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide. Species-specific ITS-RFLP profiles for *Bursaphelenchus* were generated using these five restriction enzymes.

4.2.3.3 Sequencing of D2/D3 region of 28S rDNA

The D2/D3 domain region of the 28S rDNA (LSU) was amplified in another PCR using the forward primer D2A 5'-ACA AGT ACC GTG AGG GAA AGT TG-3' and the reverse primer D3B 5'-TCG GAA GGA ACC AGC TAC TA-3' (De Ley *et al*., 1999). After purification the PCR product was ligated to a pGEM-T vector and transformed into *E. coli* strain JM109. The plasmids were purified from white colonies and sequenced (see chapter 3). The fragment was sequenced in both directions to obtain overlapping sequences of the forward and reverse DNA strand. All obtained sequences were deposited in EMBL-Genbank (Table 4.1).

For the described *Bursaphelenchus* species, the generated sequences were compared to sequences of the same species deposited in the NCBI database (28S D2D3). For the newly described species, *B. chengi*, the 28S rDNA D2/D3 sequence was compared to sequences of *Bursaphelenchus* species from different groups (*abietinus*-, *eggersi*-, *fungivorus*-, *sexdentati*- and *xylophilus*-group) deposited in the Genbank database (accession numbers in Fig 4.14).

The interspecific and intraspecific variation was estimated using the BioEdit sequence alignment editor (Hall, 1999). Alignments were calculated with ClustalW and the phylogenetic tree was constructed by the minimum evolution (ME) method of the bootstrap test and equally weighted maximum parsimony (MP) analysis using PAUP (4.0 beta version) (Swofford, 2002). *Aphelenchoides besseyi* Christie, 1942 was used as the outgroup.

4.3 Results

Five different described *Bursaphelenchus* species and one new species were identified in samples taken from 13 packaging wood samples originating from eight countries and regions (Table 4.1).

4.3.1 Characteristics of described *Bursaphelenchus* species

4.3.1.1 Morphological characterisation

Isolates BxJAP and BxCAN fitted the morphological description of *B. xylophilus*, isolates BmCAN, BmHK, BmGER, BmMEX and BmSWD conformed to the description of *B. mucronatus* Mamiya & Enda, 1979, whereas the morphology of isolates BdKOR and BdJAP corresponded to that of *B. doui* Braasch, Gu, Burgermeister & Zhang, 2004. *Bursaphelenchus xylophilus* (Figs 4.3A-B), *B. mucronatus* (Fig 4.3C) and *B. doui* (Fig 4.3D) showed the typical and strongly curved spicules of members of the *B. xylophilus* group (Ryss *et al.*, 2005) with flattened capitulum, small condylus, dorsal contour of lamina angular in posterior third, and presence of cucullus (Figs 4.3A-D); the terminal bursa was ovary-shaped (Figs 4.4A-D).

Females of the isolates belonging to these species had relatively large vulval flaps (Figs 4.1A-D). All individuals of these species showed four lines in the lateral field and had the postanal papillae typically arranged as a double pair (Braasch, 2001; Braasch *et al*., 2004a; Ryss *et al*., 2005). Isolate BxJAP had a rounded tail lacking a mucro projection, characteristic of the R-form (Fig 4.2A); the BxCAN isolate had a small mucro-like projection, hence was classified as an M-form (Fig 4.2B). *Bursaphelenchus mucronatus* has a distinct ventral mucro in all identified samples (Fig 4.2C). There were no distinct differences in female tail shape between East-Asian and European types of *B. mucronatus*.

The tail of females of the isolates BdKOR and BdJAP moderately narrowed to a slightly ventrally bent terminus with a distinct ventral mucro (Fig 4.2D), which is typical for *B. doui*. Although morphologically close, *B. doui* could be distinguished from the M-form of *B. xylophilus* and *B. mucronatus* by its larger spicules and the straight area in the central part of the lamina (Fig 4.4D).

One isolate (BtHK) was morphologically identified as *B. thailandae* Braasch & Braasch-Bidasak, 2002. It showed relatively small and delicate spicules with a darker sector and lacking a cucullus (Fig 4.3E). The isolate had four lateral lines and a relatively small stylet lacking distinct basal knobs. The female had a protruding vulval lip without vulval flap

(Fig 4.1E) and a slim tapering tail (Fig 4.2E). The male tail showed a very small terminal bursa (Fig 4.4E).

Two isolates, BrGER and BrKOR, were isolated from packaging wood originating from shipments from Germany and Korea, respectively, and identified as *B. rainulfi* Braasch & Burgermeister, 2002. Individuals from both isolates had two lateral lines. The females showed a small vulval flap (Fig 4.1F) and a slim and conoid tail with a finely rounded, ventrally bent terminus (Fig 4.2F). The spicules of the males were relatively small with a high condylus and distinct rostrum without cucullus (Fig 4.3F); a small terminal bursa was present on the mail tail (Fig 4.4F).

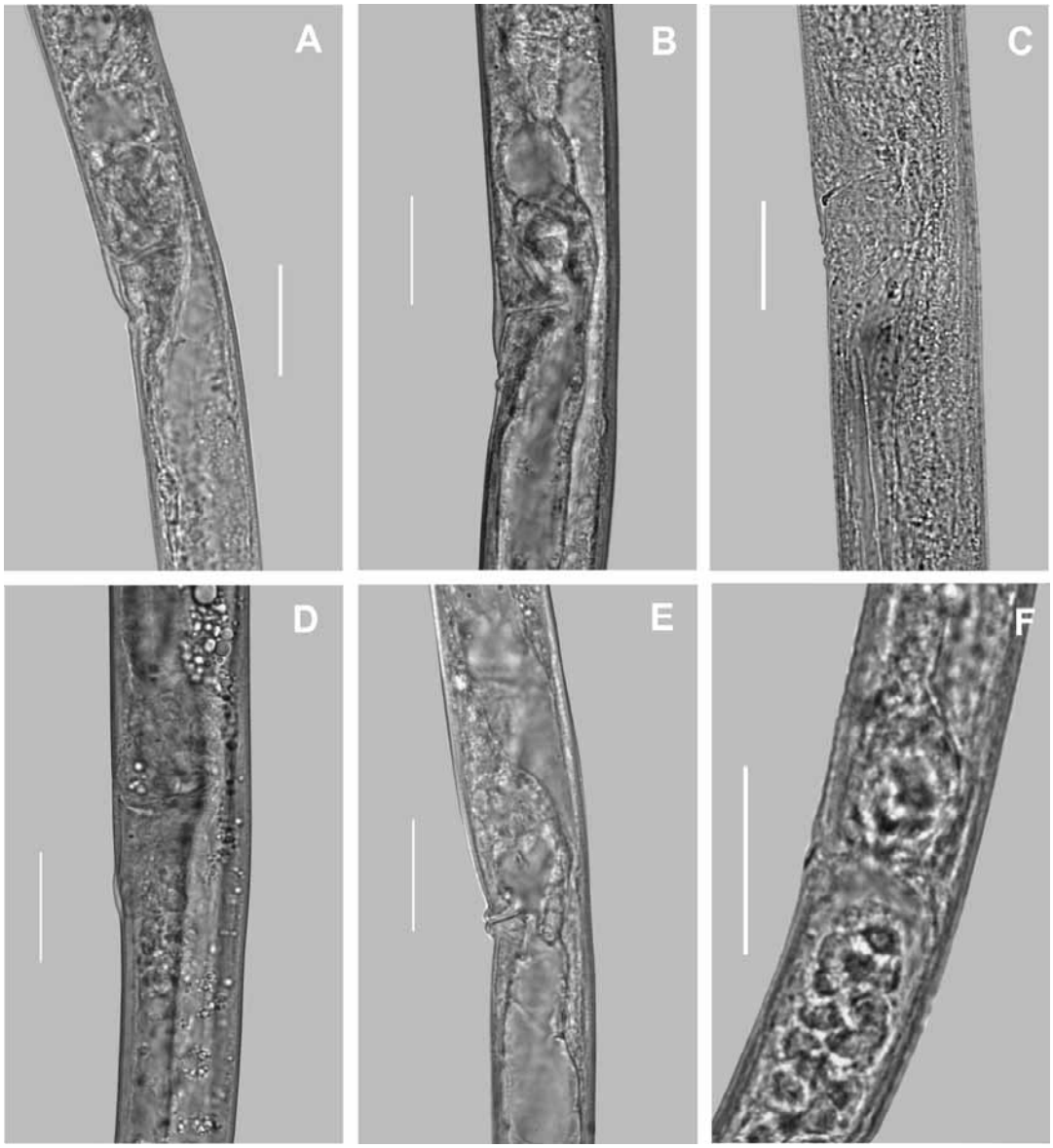

Fig 4.1 Vulva characters for five described *Bursaphelenchus* species. A: *B. xylophilus* (BxJAP), R-form; B: *B. xylophilus* (BxCAN), M-form; C: *B. mucronatus* (BmCAN); D: *B. doui* (BdKOR); E: *B. thailandae* (BtHK); F: *B. rainulfi* (BrGER) (Scale bar = 20 μm).

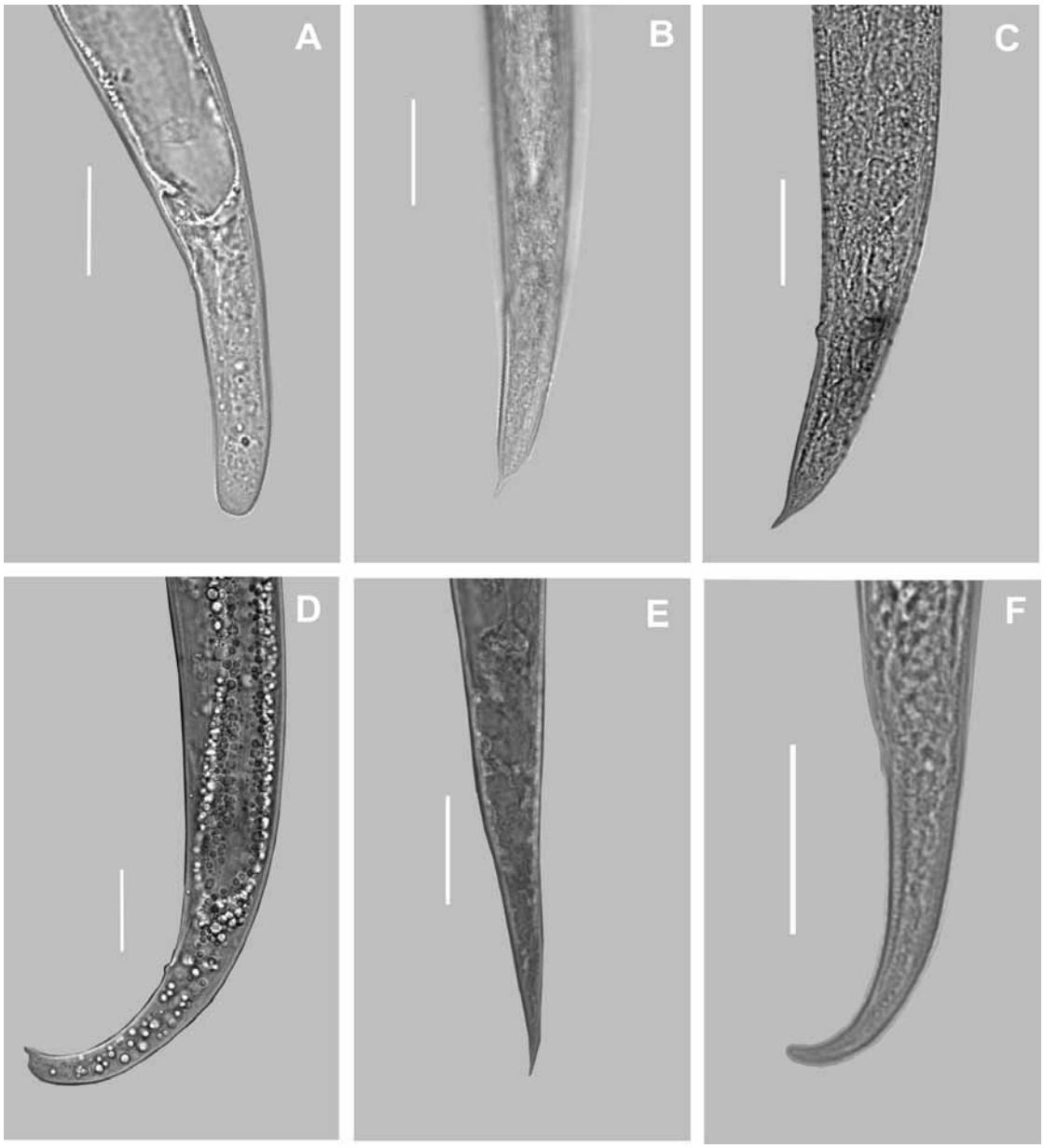

Fig 4.2 Female tail characters for five described *Bursaphelenchus* species. A: *B. xylophilus* (BxJAP), R-form; B: *B. xylophilus* (BxCAN), M-form; C: *B. mucronatus* (BmCAN); D: *B. doui* (BdKOR); E: *B. thailandae* (BtHK); F: *B. rainulfi* (BrGER) (Scale bar = 20 μm).

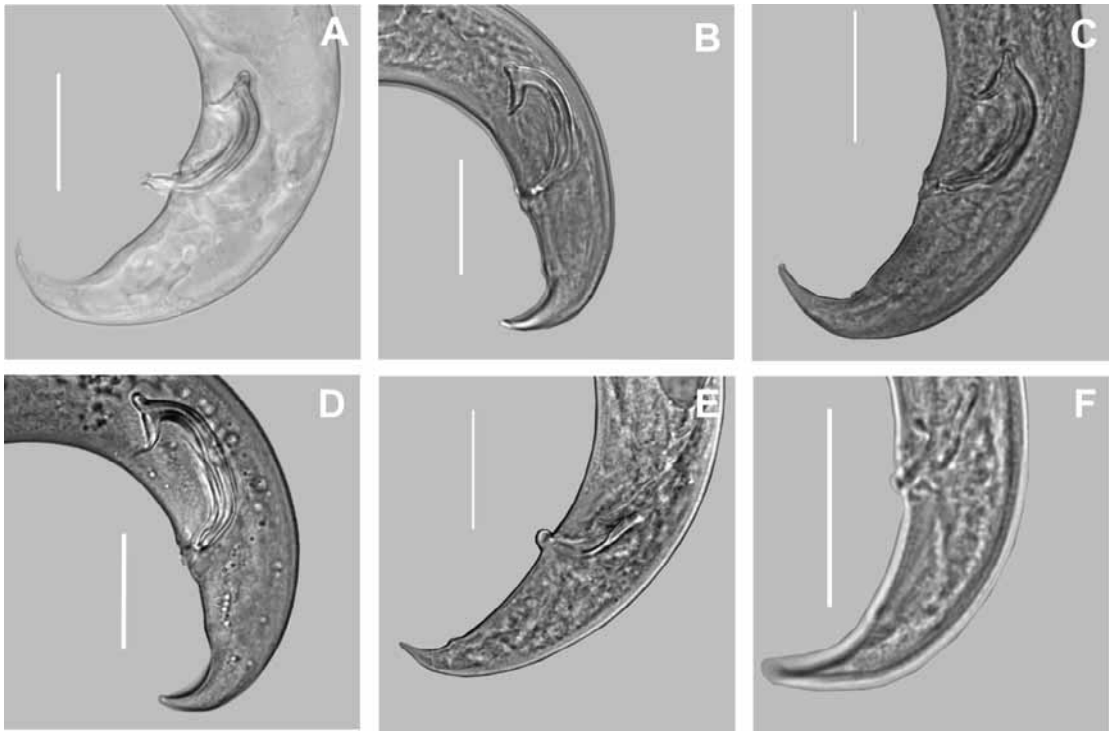

Fig 4.3 Male tail characters of five described *Bursaphelenchus* species. A: *B. xylophilus* (BxJAP), R-form; B: *B. xylophilus* (BxCAN), M-form; C: *B. mucronatus* (BmCAN); D: *B. doui* (BdKOR); E: *B. thailandae* (BtHK); F: *B. rainulfi* (BrGER) (Scale bar = 20 μm).

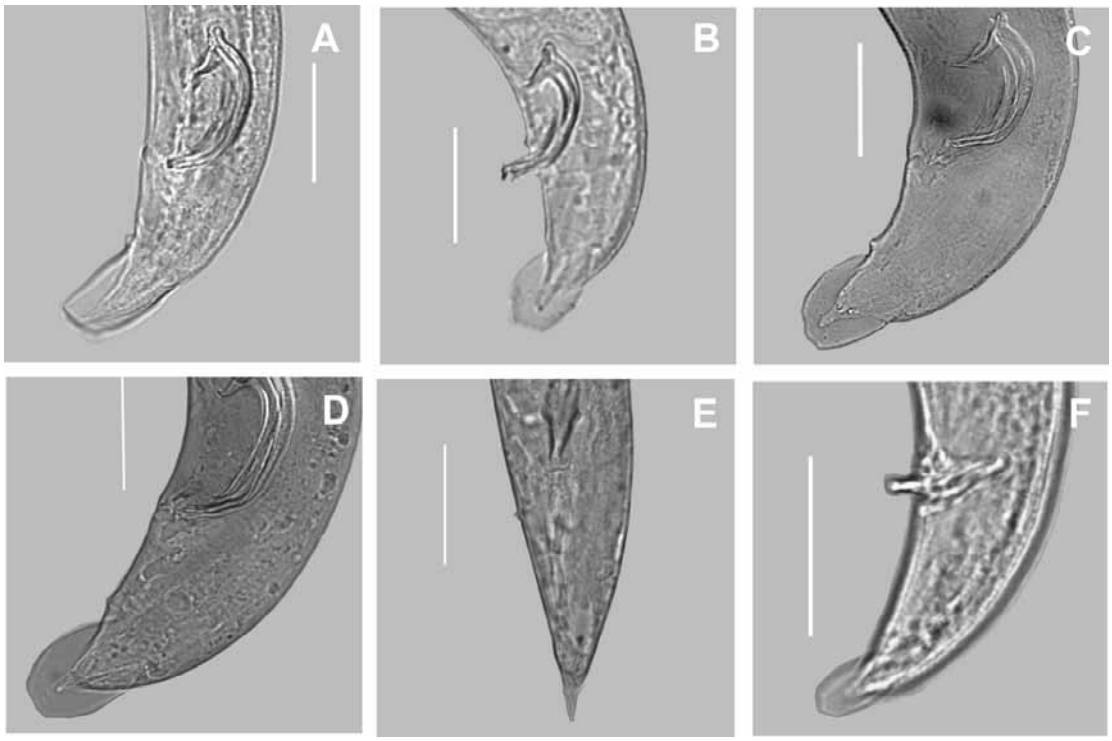

Fig 4.4 Spicule and bursa characters of five described *Bursaphelenchus* species. A: *B. xylophilus* (BxJAP), R-form; B: *B. xylophilus* (BxCAN), M-form; C: *B. mucronatus* (BmCAN); D: *B. doui* (BdKOR); E: *B. thailandae* (BtHK); F: *B. rainulfi* (BrGER) (Scale bar = 20 μm).

4.3.1.2 Morphometrical characterisation

4.3.1.2.1 *Bursaphelenchus xylophilus*

The morphometrics of the males and females of the *B. xylophilus* isolates are listed in Tables 4.2 and 4.3, respectively.

Table 4.2 Morphometric characters of males of *Bursaphelenchus xylophilus* isolates from packaging wood originating from Japan (BxJAP) and Canada (BxCAN) and from a Chinese isolate (BxLYG) kept in living collection, compared with morphometrics of type population. Measurements in μm and in form: mean ± standard deviation (range).

Characteristic	BxJAP	BxCAN	BxLYG	Japan (Mamiya & Kiyohara, 1972)
N	10	10	10	30
L	870.0±89.6 (699.2-1002.8)	765.3±93.7 (605.2-886.8)	868.0±53.2 (790.1-960.2)	730 (590-820)
a	42.8±2.4 (39.3-47.3)	44.0±4.5 (35.7-49.5)	45.6±0.9 (43.5-46.4)	42.3 (36-47)
b	11.3±0.8 (10.0-12.4)	10.2±1.1 (8.6-12.1)	11.7±0.7 (10.5-12.9)	9.4 (7.6-11.3)
b'	6.1±0.5 (5.4-6.6)	5.8±0.5 (4.6-6.6)	5.7±0.2 (5.5-6.1)	–
c	25.0±2.4 (21.1-27.7)	25.3±2.9 (21.2-29.1)	22.9±1.0 (21.5-24.6)	26.4 (21-31)
c'	2.5±0.2 (2.2-2.7)	2.4±0.3 (2.0-3.1)	2.7±0.1 (2.5-2.9)	–
Lip region diam.	7.3±0.5 (6.6-8.0)	6.6±0.4 (5.6-7.2)	7.6±0.6 (6.8-8.8)	–
Lip constriction diam.	6.3±0.4 (5.7-7.1)	6.3±0.3 (5.6-6.6)	6.5±0.4 (6.1-7.0))	–
Lip region height	3.2±0.2 (2.9-3.6)	3.1±0.2 (2.8-3.5)	3.4±0.2 (3.0-3.7)	–
Stylet	14.8±0.5 (13.5-15.4)	14.2±0.9 (12.8-15.5)	15.2±0.3 (14.6-15.6)	14.9 (14-17)
Median bulb length	17.8±0.8 (16.4-18.8)	17.3±0.9 (15.8-18.7)	18.0±1.4 (16.3-21.3)	–
Median bulb diam.	10.9±1.2 (8.7-12.3)	10.6±1.5 (8.1-12.6)	11.1±0.4 (10.2-11.7)	–
Median bulb length/median bulb diam.	1.6±0.1 (1.5-1.9)	1.7±0.2 (1.3-2.1)	1.6±0.1 (1.4-1.9)	–
Maximum body width	20.3±1.6 (17.8-23.3)	17.5±2.9 (14.6-23.6)	19.1±1.2 (17.5-21.4)	–
Body diam. at middle of median bulb	15.6±1.0 (13.9-17.4)	13.7±1.4 (11.2-16.1)	15.4±0.5 (14.7-16.1)	–
Distance from anterior end to excretory pore	95.2±5.2 (86.5-103.4)	81.9±9.0 (69.6-97.3)	92.5±3.1 (88.4-97.0)	–
Distance from anterior end to hemizonid	98.7±5.5 (90.0-106.3)	85.6±9.3 (73.3-100.5)	97.0±3.0 (93.1-101.0)	–
Distance from anterior end to distal end of median bulb	69.7±3.1 (64.5-73.1)	68.1±4.5 (61.9-75.5)	67.7±1.4 (66.3-69.2)	–
Distance from anterior end to pharynx-intestine junction	76.7±4.9 (68.2-83.0)	75.4±4.7 (67.1-83.0)	74.3±1.6 (72.3-78.1)	–
Distance from anterior end to posterior end of pharyngeal glands	142.9±10.0 (129.2-155.6)	132.0±7.7 (115.9-140.7)	152.1±8.4 (141.9-162.3)	–

Table 4.2 (Continued)

Anterior genital branch	448.5±97.6 (328.5-594.1)	327.1±52.4 (252.7-404.5)	353.8±26.3 (320.5-385.7)	–
Anal/cloacal body diam.	14.1±0.7 (12.4-14.8)	12.9±0.7 (12.0-14.1)	13.9±0.6 (13.2-15.1)	–
Tail length	34.8±2.0 (32.1-37.8)	30.5±4.4 (25.4-41.3)	37.8±1.3 (36.4-40.7)	–
T	51.5±9.4 (36.9-64.7)	43.7±10.7 (28.8-61.8)	40.8±3.0 (37.0-47.0)	–
Spicule (condylus to distal end)	25.6±1.5 (23.2-27.7)	22.9±1.2 (21.0-24.6)	24.9±1.2 (23.2-26.8)	–
Spicule (rostrum to distal end)	15.8±1.1 (14.2-17.5)	14.3±0.8 (13.4-15.7)	15.5±0.7 (14.1-16.4)	–
Spicule (curved median line)	29.2±1.6 (27.2-31.9)	26.4±1.5 (24.6-28.6)	29.4±2.0 (25.2-32.2)	27.0 (25-30)
Spicule (rostrum to condylus)	10.0±1.1 (8.9-11.5)	9.1±0.8 (7.7-10.3)	9.5±0.9 (8.0-11.1)	–
Spicule width (measured posterior to rostrum)	2.9±0.4 (2.1-3.5)	3.0±0.2 (2.5-3.3)	3.1±0.2 (2.8-3.4)	–

Table 4.3 Morphometric characters of females of *Bursaphelenchus xylophilus* isolates from packaging wood originating from Japan (BxJAP) and Canada (BxCAN) and from a Chinese population (BxLYG) kept in living collection, compared with morphometrics of type population. Measurements in μm and in form: mean ± standard deviation (range).

Characteristic	BxJAP	BxCAN	BxLYG	Japan (Mamiya & Kiyohara, 1972)
N	10	10	10	40
L	1020.6±83.2 (862.7-1112.1)	868.7±97.6 (724.5-1033.0)	943.0±51.5 (834.3-1009.4)	810 (710-1010)
a	44.7±1.3 (42.6-47.3)	47.2±3.5 (38.5-52.7)	44.1±1.6 (41.7-46.8)	40.0 (33-46)
b	12.8±0.6 (11.8-13.9)	10.8±1.2 (8.5-12.7)	12.7±0.7 (11.6-13.8)	10.3 (9.4-12.8)
b'	6.9±0.5 (6.3-8.0)	6.2±0.7 (5.1-7.1)	6.0±0.4 (5.5-6.4)	–
c	27.8±2.3 (22.4-30.6)	28.8±3.3 (24.1-36.3)	27.2±1.2 (26.0-29.4)	26.0 (23-32)
c'	3.5±0.2 (3.2-3.7)	3.3±0.3 (2.9-3.9)	3.7±0.2 (3.3-4.0)	–
V	73.6±2.2 (69.8-77.4)	74.7±2.4 (69.0-77.5)	73.7±1.1 (71.5-75.4)	72.7 (67-78)
Lip region diam.	7.2±0.3 (6.9-7.8)	6.7±0.4 (6.3-7.5)	7.7±0.3 (7.3-8.1)	–
Lip constriction diam.	6.6±0.4 (5.9-7.0)	6.0±0.5 (5.5-6.8)	6.9±0.3 (6.4-7.3)	–
Lip region height	3.2±0.3 (2.8-3.7)	3.2±0.2 (3.0-3.7)	3.6±0.4 (3.0-4.0)	–
Stylet	15.0±0.4 (14.3-15.6)	15.0±0.4 (14.6-16.0)	15.4±0.5 (14.6-16.3)	15.9 (14-18)
Median bulb length	17.9±0.9 (16.4-19.2)	18.1±1.0 (16.2-20.0)	17.9±0.9 (16.9-19.0)	–
Median bulb diam.	11.0±0.7 (10.0-12.1)	11.5±1.2 (9.8-14.1)	11.5±0.8 (10.2-12.8)	–
Median bulb length/median bulb diam.	1.6±0.1 (1.5-1.8)	1.6±0.1 (1.3-1.7)	1.6±0.1 (1.4-1.7)	–

Table 4.3 (Continued)

Maximum body width	22.8±1.5 (20.0-24.7)	18.4±1.5 (16.4-21.6)	21.4±1.6 (18.9-24.2)	–
Body diam. at middle of median bulb	16.3±0.6 (14.9-16.9)	14.6±1.2 (12.8-16.7)	15.7±0.8 (14.4-16.8)	–
Distance from anterior end to excretory pore	99.5±6.6 (90.1-111.8)	85.6±3.7 (78.5-91.9)	94.2±2.8 (89.7-98.3)	–
Distance from anterior end to Hemizonid	103.5±6.2 (93.5-114.8)	89.2±3.7 (82.6-94.0)	98.3±3.2 (93.1-102.4)	–
Distance from anterior end to distal end of median bulb	70.5±4.3 (64.8-79.0)	73.2±4.7 (64.9-82.8)	67.5±1.8 (65.1-70.7)	–
Distance from anterior end to pharynx-intestine junction	79.6±6.2 (72.8-90.8)	80.8±4.7 (72.9-88.7)	74.5±2.2 (71.7-77.4)	–
Distance from anterior end to posterior end of pharyngeal glands	148.7±12.4 (133.3-172.0)	141.0±7.7 (128.5-150.4)	158.5±8.5 (144.0-173.7)	–
Anterior genital branch	494.1±92.3 (332.6-631.7)	336.7±80.9 (236.6-487.1)	470.3±79.8 (339.5-563.6)	–
Posterior genital branch	176.0±28.8 (132.5-225.7)	110.2±16.1 (87.7-139.7)	147.7±12.1 (131.1-165.1)	–
Body diam. at vulva	22.2±1.5 (19.8-24.1)	17.8±2.0 (15.3-22.2)	21.2±1.4 (19.3-24.0)	–
Vulva to anus distance	232.9±29.0 (200.8-291.1)	187.6±15.0 (153.6-201.3)	212.8±9.0 (198.8-227.8)	–
Distance from anterior end to vulva	751.0±68.4 (623.4-838.2)	650.8±88.5 (499.7-793.1)	695.6±45.6 (603.8-761.0)	–
G1 (%)	48.2±6.9 (38.6-58.1)	38.5±6.8 (29.1-47.2)	49.8±7.6 (38.9-58.0)	–
G2 (%)	17.2±1.9 (14.8-20.8)	12.8±2.5 (10.1-18.1)	15.7±1.2 (13.0-16.7)	–
Anal/cloacal body diam.	10.5±0.4 (9.9-11.0)	9.2±0.8 (7.6-10.6)	9.3±0.3 (8.9-9.7)	–
Tail length	36.8±1.9 (34.9-40.2)	30.3±3.2 (27.5-38.6)	34.7±2.2 (31.5-37.3)	–

The male and female body length (870.0 *vs* 1020.6 μm) of the Japanese isolate was similar to those of the Chinese isolate (868.0 *vs* 943.0 μm), but longer than those of the Canadian one (765.3 *vs* 868.7 μm), which was similar to the Japanese type population (730 *vs* 810 μm) (Mamiya & Kiyohara, 1972). The spicule length (median curve line) of the males of the Japanese isolate (29.2 μm) was similar to that of the males of the Chinese isolate (29.4 μm), but longer than that of the males of the Canadian isolate (26.4 μm), which was similar to that of the males of the type population (27.0 μm) (Mamiya & Kiyohara, 1972).

Five different morphometric characters were identified as variates in the CDA of the *B. xylophilus* isolates (Table 4.4), which enabled clear separation of the isolates (Figs 4.5A-B). Three morphometrical traits of males (stylet length, c value and spicule length) provided the most useful characters for population separation; for the female, stylet length, a and c values were the best ones.

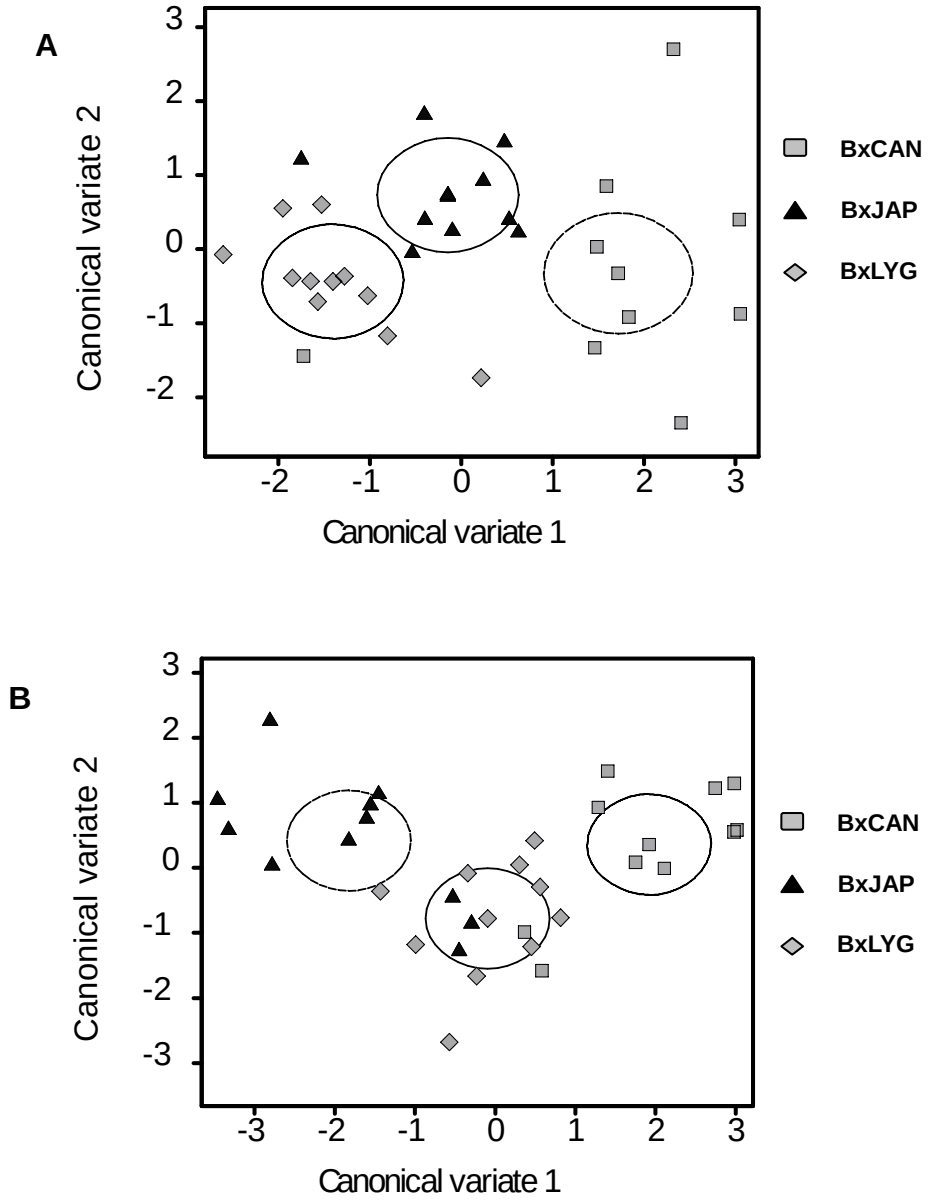

Fig 4.5 Canonical discriminant analysis of morphometric characteristics of three *Bursaphelenchus xylophilus* isolates for male (A) and female (B) performed with five variables for both (Table 4.4). The circles display 95% confidence regions.

Table 4.4 Standardised coefficients for canonical variates for males and females of three *Bursaphelenchus xylophilus* isolates.

	Males		Females	
	Root 1	Root 2	Root 1	Root 2
% of variation	85.04	14.96	88.6	11.4
Selected characters	Vector Loadings		Vector Loadings	
Body length	-0.0029	0.0011	-0.0189	0.0043
Stylet length	-1.1063	0.1327	1.6005	-2.4140
a	-0.0076	-0.2750	0.3001	0.1326
c	0.3927	0.2322	0.1612	0.0027
V			0.0307	0.1651
Spicule length	-0.2917	0.2119		

4.3.1.2.2 *Bursaphelenchus mucronatus*

The morphometrics of males and females of the *B. mucronatus* isolates, such as the length of body, spicule and stylet, were within the range of the type population (Mamiya & Enda, 1979) (Tables 4.5 and 4.6). The only apparent difference with the type population was the c value of males (21.5-24.2 vs 25.7-35.6). Five different morphometric characters were used as variates in the CDA of males and females of *B. mucronatus* (Table 4.7).

Using the male characters, the CDA clearly separated the six isolates into two groups (Fig 4.6A). Three morphometrical traits of males (spicule length, stylet length and c value) provided the most useful taxonomic information. CDA of females did not reveal a separation of the six isolates (Fig 4.6B).

Table 4.7 Standardised coefficients for canonical variates for males and females of six *Bursaphelenchus mucronatus* isolates.

	Males		Females	
	Root 1	Root 2	Root 1	Root 2
% of variation	51.64	34.31	71.43	17.35
Selected characters	Vector Loadings		Vector Loadings	
Body length	0.0095	0.0018	0.0021	0.0148
Stylet length	0.2308	1.5857	1.0623	-0.8709
a	-0.1083	-0.4603	-0.4809	-0.1139
c	0.2287	-0.1375	-0.0039	-0.4011
V			-0.0479	0.4677
Spicule length	-0.8999	-0.1194		

Table 4.5 Morphometric characters of males of *Bursaphelenchus mucronatus* isolates from packaging wood originating from Germany (BmGER), Sweden (BmSWD), Mexico (BmMEX), Canada (BmCAN) and Hongkong (BmHK) and from a Chinese population (BmNJ) kept in living collection, compared with morphometrics of type population. Measurements in μm and in form: mean standard ± deviation (range).

Characteristic	BmGER	BmSWD	BmMEX	BmCAN	BmHK	BmNJ	Japan (Mamiya & Enda, 1979)
N	10	10	10	10	10	10	35
L	862.9±59.0 (709.9-906.1)	766.7±53.6 (679.7-853.2)	788.4±86.2 (698.1-980.1)	804.5±80.0 (692.3-930.0)	801.2±44.7 (746.8-891.0)	831.8±53.5 (751.1-910.2)	790 (640-970)
a	43.2±1.7 (39.3-45.0)	38.4±2.6 (32.7-41.4)	44.0±2.2 (41.1-46.8)	42.8±2.0 (39.5-45.8)	42.6±1.0 (41.5-44.4)	43.3±1.9 (39.7-46.0)	44.0 (38.8-51.1)
b	11.6±0.9 (9.5-12.7)	11.2±0.7 (10.0-12.3)	11.7±1.2 (10.1-14.0)	11.3±1.1 (9.8-12.9)	12.0±1.0 (11.1-14.2)	11.2±0.8 (10.0-12.3)	11.4 (9.0-14.7)
b'	6.6±0.5 (5.6-7.3)	5.4±0.3 (4.9-6.1)	6.5±0.8 (5.3-7.9)	6.3±0.4 (5.6-6.9)	6.5±0.5 (6.0-7.4)	6.0±0.3 (5.5-6.5)	–
c	23.0±1.1 (21.4-25.0)	21.5±1.3 (18.9-23.8)	22.1±1.8 (19.5-24.2)	24.2±1.4 (22.5-26.6)	23.2±1.1 (21.7-25.2)	23.1±0.7 (21.6-23.9)	29.1 (25.7-35.6)
c'	2.6±0.1 (2.3-2.7)	2.4±0.2 (2.1-2.7)	2.48±0.1 (2.3-2.8)	2.5±0.1 (2.3-2.7)	2.5±0.1 (2.3-2.6)	2.7±0.1 (2.5-2.9)	–
Lip region diam.	7.6±0.5 (6.9-8.4)	7.3±0.4 (6.8-8.1)	7.5±0.6 (6.7-8.8)	7.5±0.5 (7.0-8.6)	7.8±0.4 (7.3-8.4)	7.6±0.5 (6.9-8.5)	–
Lip constriction diam.	6.8±0.4 (6.3-7.5)	6.5±0.3 (6.1-7.2)	6.5±0.5 (6.1-7.4)	6.5±0.5 (6.0-7.8)	6.6±0.4 (6.1-7.1)	6.4±0.5 (5.6-7.5)	–
Lip region height	3.7±0.3 (3.2-4.0)	3.5±0.3 (3.2-3.9)	3.5±0.3 (3.0-4.0)	3.4±0.3 (3.0-4.1)	3.5±0.2 (3.1-3.9)	3.7±0.3 (3.4-4.3)	–
Stylet	15.7±0.4 (15.2-16.5)	15.6±0.4 (15.1-16.4)	14.9±0.4 (14.4-15.7)	15.2±0.6 (13.7-15.9)	15.6±0.3 (15.2-16.0)	15.1±0.4 (14.6-15.6)	15.0 (14-16)
Median bulb length	17.5±1.0 (16.2-19.1)	16.4±1.2 (15.0-18.6)	17.7±0.7 (16.2-18.9)	17.3±1.1 (14.5-18.4)	17.0±0.8 (15.3-18.4)	17.7±0.8 (16.0-18.5)	–
Median bulb diam.	11.9±0.9 (10.8-13.5)	11.6±1.0 (10.3-13.6)	11.7±0.7 (10.5-12.8)	11.6±1.2 (9.3-13.3)	11.6±0.5 (10.8-12.2)	11.1±0.7 (10.2-11.9)	–
Median bulb length /median bulb diam.	1.5±0.1 (1.4-1.5)	1.4±0.1 (1.3-1.5)	1.5±0.1 (1.4-1.7)	1.5±0.1 (1.4-1.7)	1.5±0.1 (1.3-1.6)	1.6±0.1 (1.5-1.8)	–
Maximum body width	20.0±1.1 (18.1-21.5)	20.0±1.7 (17.4-22.7)	17.9±1.8 (15.2-21.2)	18.8±1.2 (16.6-20.7)	18.3±1.0 (17.5-20.8)	19.2±1.2 (17.2-20.8)	–
Body diam. at middle of median bulb	16.0±1.1 (14.3-17.6)	16.0±1.0 (14.4-17.6)	15.6±0.8 (14.3-16.7)	14.9±1.0 (12.9-16.3)	15.5±0.7 (14.7-16.7)	15.3±0.9 (14.4-17.5)	–
Distance from anterior end to excretory pore	88.7±2.4 (84.8-92.7)	90.0±3.0 (84.5-93.7)	84.1±6.1 (76.1-95.3)	89.3±5.0 (80.6-16.3)	85.0±3.4 (80.0-91.1)	92.7±4.2 (85.5-98.0)	–

Table 4.5 (Continued)

Distance from anterior end to Hemizonid	93.2±2.4 (84.8-92.7)	94.1±2.6 (90.1-97.7)	88.5±6.0 (80.7-100.1)	93.2±5.5 (83.9-101.5)	89.0±3.2 (84.5-94.5)	97.0±4.6 (90.1-103.1)	–
Distance from anterior end to distal end of median bulb	68.4±1.7 (65.3-71.9)	68.7±2.6 (64.0-73.7)	60.9±3.2 (55.2-66.1)	66.3±3.4 (51.7-70.9)	59.7±4.2 (52.0-67.4)	68.1±2.3 (65.4-72.5)	–
Distance from anterior end to pharynx-intestine junction	74.2±1.9 (71.2-77.2)	75.0±2.8 (71.5-81.7)	67.2±3.3 (60.9-70.8)	71.3±3.2 (66.1-76.6)	66.9±4.0 (59.4-74.5)	74.3±1.8 (71.3-77.6)	–
Distance from anterior end to posterior end of pharyngeal glands	130.7±5.1 (123.8-139.0)	142.2±7.9 (130.2-154.2)	121.2±8.6 (106.7-134.8)	127.9±7.1 (117.0-137.1)	123.2±8.7 (113.1-139.5)	139.7±6.9 (129.6-147.9)	–
Anterior genital branch	454.2±45.8 (380.6-516.7)	430.6±64.5 (288.6-508.1)	384.6±60.0 (301.1-505.0)	351.2±32.6 (301.3-395.8)	380.6±42.3 (314.2-441.3)	389.2±43.7 (328.4-447.2)	–
Anal/cloacal body diam.	14.7±1.0 (13.11-16.3)	15.0±1.4 (12.9-16.8)	14.4±1.0 (12.5-16.0)	13.2±0.9 (12.0-14.7)	13.8±0.6 (13.0-15.1)	13.6±1.0 (11.9-14.8)	–
Tail length	37.6±3.0 (31.5-40.6)	35.7±2.0 (33.5-39.5)	35.6±2.5 (32.6-40.3)	33.2±2.1 (29.7-35.7)	34.5±1.5 (32.5-36.9)	36.1±2.4 (32.8-39.5)	–
T	52.7±4.1 (46.3-59.0)	55.9±5.8 (42.5-62.4)	48.8±5.3 (42.6-58.4)	43.8±3.4 (40.6-51.3)	47.6±5.8 (37.2-56.7)	46.7±3.7 (42.8-52.4)	–
Spicule (condylus to distal end)	25.5±1.2 (23.9-27.3)	24.1±1.0 (22.3-25.3)	24.1±1.2 (21.3-25.4)	22.3±1.5 (19.9-25.2)	22.9±0.7 (21.6-24.1)	23.4±1.1 (21.5-25.0)	–
Spicule (rostrum to distal end)	15.9±0.5 (15.0-16.5)	15.1±0.8 (14.0-16.2)	15.0±0.9 (13.7-16.8)	14.0±1.1 (12.6-15.6)	14.1±0.6 (13.3-15.6)	14.6±1.1 (13.1-16.4)	–
Spicule (curved median line)	30.6±1.5 (27.8-33.0)	29.1±0.9 (27.5-30.8)	29.6±1.2 (27.1-30.6)	26.6±1.9 (23.8-29.7)	26.8±1.0 (25.5-28.9)	27.5±1.4 (25.6-30.2)	26.0 (23-29)
Spicule (rostrum to condylus)	10.15±0.8 (9.4-11.7)	9.2±0.8 (8.2-10.6)	9.2±0.8 (7.9-10.1)	8.7±0.4 (8.4-9.6)	9.3±0.3 (8.8-9.7)	9.0±1.1 (7.1-10.6)	–
Spicule width (measured posterior to rostrum)	2.6±0.2 (2.2-2.9)	2.3±0.4 (1.9-3.1)	2.2±0.3 (1.8-2.8)	2.3±0.3 (2.0-2.7)	2.4±0.3 (2.1-2.9)	2.4±0.3 (2.0-2.8)	–

Table 4.6 Morphometric characters of females of *Bursaphelenchus mucronatus* isolates from packaging wood originating from Germany (BmGER), Sweden (BmSWD), Mexico (BmMEX), Canada (BmCAN) and Hongkong (BmHK) and from a Chinese population (BmNJ) kept in living collection, compared with morphometrics of type population. Measurements in μm and in form: mean standard ± deviation (range).

Characteristic	BmGER	BmSWD	BmMEX	BmCAN	BmHK	BmNJ	Japan (Mamiya & Enda, 1979)
n	10	10	10	10	10	10	40
L	931.2±73.8 (794.1-1033.7)	882.3±61.4 (804.1-989.3)	892.3±75.5 (814.1-1018.4)	876.4±98.8 (718.6-1002.7)	931.0±52.6 (840.9-986.7)	932.7±57.5 (844.6-1004.2)	870 (700-980)
a	42.8±2.7 (39.1-46.5)	38.9±2.1 (35.4-43.8)	44.7±3.0 (41.7-51.1)	43.5±1.8 (41.1-45.9)	43.9±1.4 (41.2-46.4)	44.0±1.6 (41.7-46.2)	41.8 (36.5-45.9)
b	12.5±1.3 (10.2-14.8)	11.7±0.9 (10.4-13.4)	13.4±1.0 (12.2-15.1)	12.9±1.8 (10.1-14.8)	13.0±0.8 (11.8-14.1)	12.4±0.8 (11.5-13.4)	12.6 (9.6-15.9)
b'	6.6±0.5 (5.6-7.2)	5.9±0.5 (5.2-7.1)	7.2±0.5 (6.3-8.2)	7.1±1.1 (5.4-8.6)	7.2±0.3 (6.9-7.7)	6.6±0.4 (6.1-7.2)	–
c	27.0±2.1 (22.8-30.8)	27.4±2.7 (23.0-33.5)	28.6±2.8 (25.7-35.3)	27.9±2.1 (25.0-31.7)	28.2±2.3 (25.4-32.0	27.0±2.3 (23.7-30.6)	26.2 (19.6-30.4)
c'	3.3±0.3 (2.8-4.0)	3.3±0.2 (2.9-3.5)	3.8±0.5 (3.0-4.5)	3.5±0.5 (3.0-4.3)	3.4±0.3 (3.1-4.0)	3.8±0.3 (3.3-4.1)	–
V	73.5±0.7 (72.6-74.6)	73.9±1.7 (70.2-76.4)	74.1±1.3 (72.0-76.5)	74.5±2.7 (67.7-77.5)	73.9±1.0 (72.0-75.5)	74.7±0.8 (73.5-76.2)	75 (73-77)
Lip region diam.	8.1±0.4 (7.5-8.7)	7.8±0.5 (7.0-8.4)	7.8±0.4 (7.4-8.6)	7.7±0.7 (6.7-8.6)	7.6±0.3 (6.9-8.3)	7.8±0.3 (7.1-8.2)	–
Lip constriction diam.	7.2±0.4 (6.4-7.7)	7.1±0.4 (6.6-7.9)	7.1±0.2 (6.7-7.4)	6.8±0.7 (5.8-7.7)	7.0±0.3 (6.7-7.5)	6.5±0.4 (5.8-7.2)	–
Lip region height	3.6±0.2 (3.3-3.9)	3.6±0.2 (3.4-4.0)	3.4±0.2 (3.1-3.8)	3.5±0.2 (3.2-3.8)	3.5±0.3 (3.1-3.8)	3.6±0.2 (3.2-3.8)	–
Stylet	15.9±0.5 (15.3-16.7)	15.8±0.4 (15.2-16.6)	15.4±0.6 (14.7-16.2)	15.3±0.7 (14.5-16.7)	15.7±0.3 (15.4-16.3)	15.3±0.6 (14.7-16.7)	15.8 (14-16)
Median bulb length	18.9±1.2 (16.7-20.3)	18.2±0.4 (17.4-18.8)	18.0±1.0 (16.5-19.7)	17.5±1.0 (15.2-18.6)	17.9±0.7 (16.8-19.2)	18.1±1.1 (16.2-19.3)	–
Median bulb diam.	12.5±1.0 (10.8-13.6)	12.7±0.7 (11.8-14.3)	11.7±0.9 (10.5-13.4)	11.5±1.1 (10.2-13.3)	12.1±0.5 (11.2-13.0)	11.3±0.8 (10.2-12.9)	–
Median bulb length/median bulb diam.	1.5±0.1 (1.3-1.7)	1.4±0.1 (1.3-1.5)	1.5±0.1 (1.4-1.8)	1.5±0.1 (1.4-1.7)	1.5±0.1 (1.3-1.6)	1.6±0.1 (1.5-1.8)	–
Maximum body width	21.7±1.1 (19.7-23.0)	22.7±1.3 (21.4-24.9)	20.0±1.1 (19.0-22.8)	20.1±2.1 (16.8-22.8)	21.2±1.4 19.0-23.8)	21.1±1.7 (18.5-22.9)	–
Body diam. at middle of median bulb	17.1±1.0 (15.1-18.2)	17.5±0.6 (16.6-18.5)	16.2±1.0 (14.8-17.9)	14.9±1.0 (13.5-16.7)	16.5±0.7 (15.5-17.6)	15.7±0.6 (14.5-16.5)	–

Table 4.6 (Continued)

Distance from anterior end to excretory pore	91.9±3.5 (86.7-96.9)	90.1±2.6 (85.8-94.1)	85.8±3.4 (80.0-90.1)	90.8±3.6 (86.4-99.0)	90.3±4.8 (82.2-96.7)	94.8±3.2 (89.5-97.6)	–
Distance from anterior end to Hemizonid	95.8±3.3 (90.4-100.3)	94.2±2.6 (89.5-98.3)	90.1±3.6 (84.2-94.8)	95.4±3.8 (90.1-104.0)	93.9±5.1 (85.5-100.6)	99.1±3.6 (93.6-104.1)	–
Distance from anterior end to distal end of median bulb	68.5±5.4 (54.7-72.7)	69.9±2.0 (67.3-72.6)	59.7±2.0 (56.7-62.7)	62.6±2.8 (59.5-67.1)	64.3±2.1 (60.5-67.0)	68.4±2.0 (66.2-72.5)	–
Distance from anterior end to pharynx-intestine junction	74.8±6.1 (59.3-79.6)	75.7±2.5 (72.3-79.1)	66.4±2.7 (62.5-71.1)	68.2±3.1 (64.4-73.9)	71.5±1.6 (70.0-74.5)	75.3±3.0 (73.0-82.5)	–
Distance from anterior end to posterior end of pharyngeal glands	140.6±6.2 (129.8-147.5)	150.1±7.7 (139.0-161.7)	124.2±7.6 (112.0-137.8)	123.7±8.7 (110.2-140.3)	129.1±5.9 (121.5-137.1)	141.6±7.1 (132.9-153.2)	–
Anterior genital branch	386.3±63.9 (296.9-469.4)	350.4±55.1 (284.1-433.1)	360.3±44.3 (298.7-439.1)	346.7±63.0 (258.2-438.4)	559.2±40.5 (483.8-601.8)	361.3±39.5 (317.2-425.2)	–
Posterior genital branch	150.6±15.4 (127.1-177.6)	140.1±16.8 (116.3-169.4)	134.0±13.3 (112.3-151.3)	120.1±24.7 (82.2-155.3)	148.7±8.0 (138.7-163.3)	121.1±25.0 (86.2-153.7)	–
Body diam. at vulva	21.6±1.2 (19.9-23.2)	21.8±0.8 (20.8-22.8)	20.0±0.7 (19.2-21.1)	18.8±1.3 (16.8-21.4)	20.1±1.2 (18.2-21.6)	19.8±1.2 (17.9-21.5)	–
Vulva to anus distance	212.2±17.5 (182.9-234.6)	197.2±16.5 (177.0-229.7)	199.7±17.2 (175.7-230.5)	192.7±36.5 (150.9-282.8)	209.6±9.5 (198.0-225.6)	201.1±16.3 (170.0-219.8)	–
Distance from anterior end to vulva	684.4±55.0 (576.4-757.7)	652.8±53.1 (585.9-756.3)	661.3±62.1 (591.5-779.0)	652.2±73.3 (527.0-753.3)	688.3±44.1 (605.3-735.1)	696.8±41.3 (631.9-752.2)	–
G1 (%)	41.3±4.6 (35.4-48.9)	39.6±4.3 (34.1-45.5)	40.4±3.2 (36.7-47.4)	39.3±3.3 (34.6-44.5)	60.0±1.5 (57.5-62.5)	38.8±3.8 (33.7-46.7)	–
G2 (%)	16.2±0.8 (14.8-17.8)	15.9±1.5 (14.2-19.2)	15.1±1.6 (12.4-18.4)	13.6±2.1 (10.6-17.3)	16.0±0.8 (14.7-16.8)	12.9±2.3 (10.0-16.2)	–
Anal/cloacal body diam.	10.4±0.8 (8.8-11.8)	10.0±0.5 (8.9-10.6)	8.3±1.0 (7.1-9.5)	9.0±0.8 (7.6-10.1)	9.7±0.7 (8.8-10.9)	9.2±0.7 (8.2-10.3)	–
Tail length	34.6±3.3 (28.5-41.4)	32.4±2.3 (29.5-35.8)	31.3±1.6 (28.8-33.4)	31.4±3.3 (26.9-37.8)	33.1±3.3 (29.0-38.6)	34.8±3.8 (29.1-41.7)	–

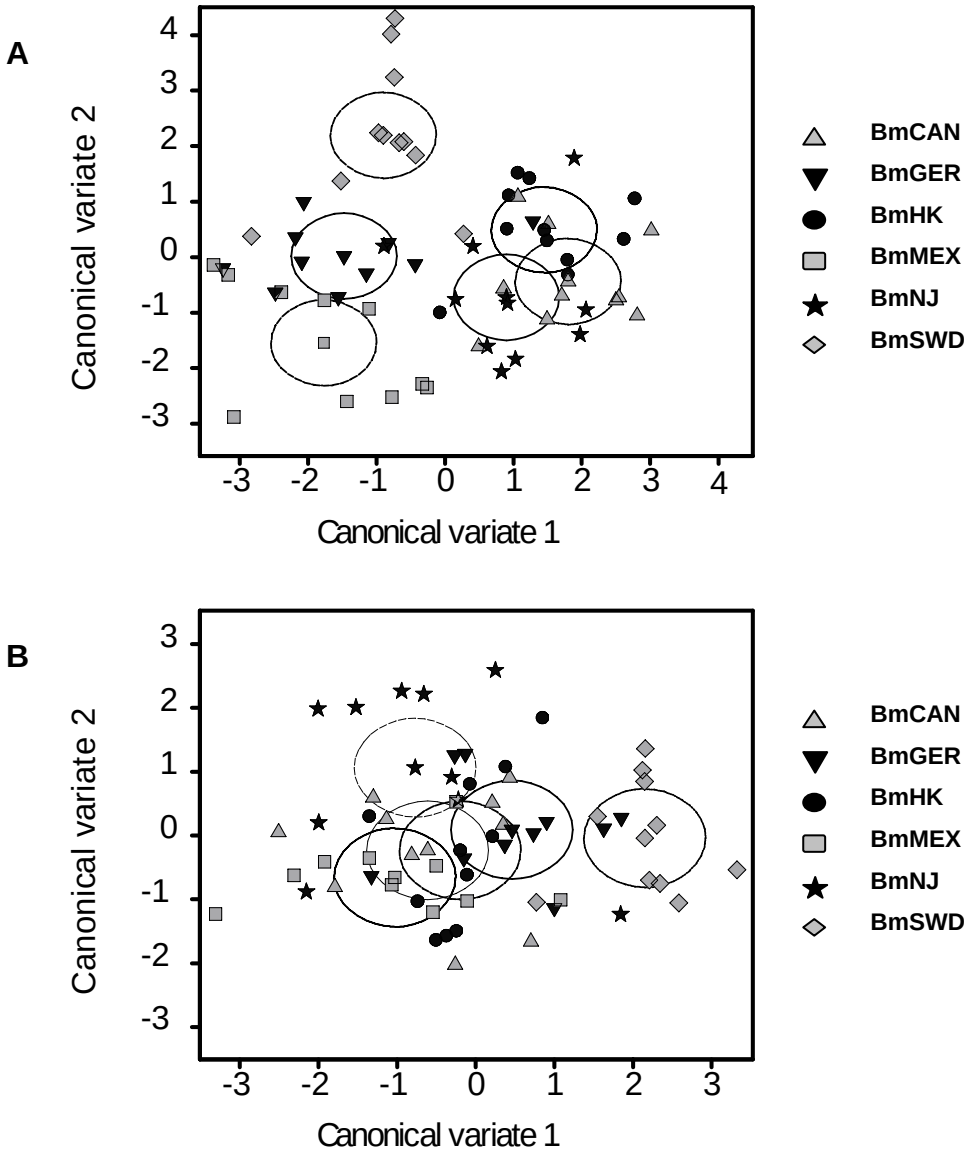

Fig 4.6 Canonical discriminant analysis of morphometric characteristics of six *Bursaphelenchus mucronatus* isolates for male (A) and female (B) performed with five variables for both (Table 4.7). The circles display 95% confidence regions.

4.3.1.2.3 *Bursaphelenchus doui*

The morphometrics of males and females of the *B. doui* isolates are listed in Tables 4.8 and 4.9, respectively. The average body length of males and females of the Korean (606 and 721 μm, respectively), Japanese (672 and 816 μm, respectively) and Chinese (738 and 848 μm, respectively) isolates were shorter than those of the type population (811 and 876 μm, respectively) (Braasch *et al*., 2004a). The mean spicule length of males of the type population (33.8-43.3 μm) was longer than that of the males of the three isolates (33.0-36.8 μm). The spicule length of *B. doui* males was longer than that of males of *B. xylophilus* (24.6-32.2 μm) and *B. mucronatus* (23.0-30.2 μm).

Table 4.8 Morphometric characters of males of *Bursaphelenchus doui* isolates from packaging wood originating from South Korea (BdKOR) and Japan (BdJAP) and from a Chinese population (BdZJ) kept in living collection, compared with morphometrics of type population. Measurements in μm and in form: mean standard ± deviation (range).

Characteristic	BdKOR	BdJAP	BdZJ	Taiwan or Korea (Braasch *et al*., 2004a)
n	15	10	10	15
L	606.4±35.4 (558.6-692.0)	671.5±62.0 (529.0-739.1)	737.6±43.7 (682.2-803.8)	811.3±84.1 (629.0-948.0)
a	33.6±1.2 (31.4-35.5)	33.9±2.6 (29.4-37.4)	33.5±2.8 (28.4-36.8)	28.8±4.5 (25.0-33.0)
b	7.9±0.5 (6.9-8.9)	8.8±0.7 (7.6-9.6)	10.0±0.8 (9.2-11.8)	8.4±0.8 (6.3-9.7)
b'	4.4±0.4 (3.8-5.3)	5.1±0.5 (4.3-5.9)	6.0±0.3 (5.5-6.6)	–
c	18.2±1.9 (12.5-20.4)	20.8±2.1 (18.8-26.3)	21.1±1.3 (19.7-23.1)	21.9±3.4 (17.5-30.7)
c'	2.2±0.2 (2.0-2.8)	2.2±0.2 (1.8-2.6)	2.1±0.1 (1.9-2.3)	1.9±0.2 (1.5-2.3)
Lip region diam.	7.3±0.4 (6.7-8.0)	7.3±0.7 (6.3-8.4)	7.9±0.5 (7.3-8.8)	–
Lip constriction diam.	6.6±0.4 (5.6-7.2)	6.5±0.8 (5.2-7.9)	6.9±0.4 (6.3-7.6)	–
Lip region height	3.4±0.3 (3.0-3.8)	3.6±0.3 (3.1-4.0)	3.8±0.3 (3.5-4.3)	–
Stylet	14.8±0.4 (14.2-15.6)	14.9±0.6 (13.7-15.9)	15.5±0.6 (14.6-16.1)	15.2±0.7 (14.1-15.9)
Median bulb length	16.8±1.0 (15.5-18.6)	16.5±1.2 (14.7-18.2)	18.0±0.6 (16.8-18.6)	–
Median bulb diam.	12.7±1.0 (11.3-15.2)	11.7±1.3 (10.0-13.7)	13.2±0.7 (11.8-14.2)	–
Median bulb length/median bulb diam.	1.3±0.1 (1.1-1.5)	1.4±0.1 (1.3-1.6)	1.4±0.1 (1.3-1.4)	–
Maximum body width	18.0±0.7 (17.0-19.5)	19.9±1.7 (17.1-21.9)	22.2±2.1 (19.8-25.1)	–
Body diam. at middle of median bulb	15.9±0.6 (14.8-16.9)	15.8±1.5 (14.1-17.8)	16.9±0.9 (15.3-18.5)	–
Distance from anterior end to excretory pore	82.6±5.1 (71.1-94.2)	89.7±5.8 (81.3-97.1)	88.6±4.6 (82.5-98.4)	–
Distance from anterior end to hemizonid	85.8±4.2 (78.6-95.8)	93.8±6.0 (85.0-101.2)	93.4±4.9 (86.2-103.0)	–
Distance from anterior end to distal end of median bulb	63.9±3.0 (57.1-68.0)	68.6±3.7 (61.7-73.8)	66.4±4.3 (58.8-71.2)	–
Distance from anterior end to pharynx-intestine junction	77.1±4.0 (71.2-87.3)	76.4±4.2 (69.5-83.3)	74.0±3.0 (67.7-77.1)	–
Distance from anterior end to posterior end of pharyngeal glands	137.3±9.9 (119.1-152.3)	131.6±8.8 (122.7-150.2)	123.9±5.9 (113.8-130.6)	–
Anterior genital branch	265.7±48.4 (108.0-416.3)	362.0±24.8 (331.3-404.3)	318.8±64.0 (221.3-439.8)	–
Anal/cloacal body diam.	15.5±1.0 (13.9-17.5)	15.0±1.2 (13.6-17.6)	16.9±1.0 (15.5-18.3)	–
Tail length	33.6±3.4 (29.0-44.6)	32.5±2.8 (27.2-35.4)	35.0±2.0 (31.7-38.6)	–
T	43.6±5.7 (36.6-60.2)	54.2±4.9 (44.8-62.7)	43.1±7.7 (32.4-54.7)	–
Spicule (condylus to distal end)	27.6±1.6 (25.4-30.5)	28.0±2.3 (24.3-31.0)	31.9±0.9 (30.5-33.5)	–
Spicule (rostrum to distal end)	18.6±1.4 (15.6-20.7)	18.3±1.4 (16.5-19.9)	20.5±0.9 (18.6-21.8)	–

Table 4.8 (Continued)

Spicule (curved median line)	33.0±2.3 (28.4-36.1)	33.1±3.6 (27.9-37.4)	36.8±1.3 (35.5-39.4)	39.6±2.5* (33.8-43.3)
Spicule (rostrum to condylus)	9.5±0.7 (8.1-10.6)	10.0±1.2 (7.4-11.4)	11.5±0.9 (10.2-12.8)	–
Spicule width (measured posterior to rostrum)	3.5±0.3 (2.8-4.2)	3.1±0.3 (2.8-3.8)	3.0±0.4 (2.4-3.7)	–

* Spicules measured across bow.

Table 4.9 Morphometric characters of females of *Bursaphelenchus doui* isolates from packaging wood originating from South Korea (BbKOR) and Japan (BdJAP) and from a Chinese population (BdZJ) kept in living collection, compared with morphometrics of type population. Measurements in μm and in form: mean ± standard deviation (range).

Characteristic	BbKOR	BdJAP	BdZJ	Taiwan or Korea (Braasch *et al.,* 2004a)
n	15	10	10	15
L	721.1±59.5 (617.1-822.9)	816.1±70.0 (685.3-934.1)	848.0±102.9 (678.5-981.8)	875.9±112.9 (634-1143)
a	33.9±1.4 (30.4-35.7)	35.8±3.4 (32.0-42.4)	36.7±2.2 (33.3-40.9)	32.6±3.5 (26.6-37.3)
b	8.6±0.7 (7.6-9.7)	10.5±1.0 (8.6-11.7)	10.8±1.0 (9.2-12.3)	9.5±1.8 (6.5-12.9)
b'	5.2±0.5 (4.4-6.3)	6.5±0.6 (5.4-7.3)	6.3±0.4 (5.6-6.8)	–
c	22.8±1.6 (20.0-26.0)	23.7±2.1 (21.8-28.7)	25.3±1.7 (21.5-27.3)	23.4±2.2 (19.7-28.6)
c'	3.50±0.2 (3.1-3.9)	3.5±0.3 (3.1-4.0)	3.6±0.3 (3.2-4.0)	3.6±0.3 (2.8-4.2)
V	74.2±1.9 (71.7-78.2)	75.5±0.5 (74.6-76.1)	75.1±1.0 (73.5-76.4)	74.9±1.7 (72.1-78.3)
Lip region diam.	7.7±0.4 (7.0-8.7)	7.6±0.5 (6.9-8.4)	8.0±0.7 (6.7-9.0)	–
Lip constriction diam.	7.1±0.6 (6.2-7.8)	6.7±0.7 (5.6-7.5)	7.3±0.7 (5.8-8.0)	–
Lip region height	3.5±0.2 (3.1-3.9)	3.6±0.3 (3.1-4.2)	3.7±0.4 (2.8-4.1)	–
Stylet	14.9±0.5 (14.3-15.9)	15.1±0.6 (13.8-15.6)	15.4±0.4 (14.8-16.2)	15.1±0.8 (13.0-16.2)
Median bulb length	17.8±1.2 (15.9-20.4)	17.5±0.9 (15.9-19.0)	18.2±0.9 (16.3-19.4)	–
Median bulb diam.	13.4±1.1 (10.8-15.7)	13.1±1.2 (10.9-15.1)	13.3±1.1 (11.4-14.7)	–
Median bulb length/median bulb diam.	1.3±0.1 (1.2-1.5)	1.3±0.1 (1.2-1.5)	1.4±0.1 (1.3-1.6)	–
Maximum body width	21.3±1.8 (19.1-24.7)	22.9±2.2 (19.7-25.5)	23.1±2.6 (18.9-27.5)	–
Body diam. at middle of median bulb	17.3±1.0 (15.3-18.8)	16.7±1.5 (14.0-18.6)	17.4±1.0 (15.7-18.5)	–
Distance from anterior end to excretory pore	88.4±3.7 (81.9-95.6)	96.1±6.7 (78.1-103.0)	93.5±5.7 (85.6-102.5)	–
Distance from anterior end to Hemizonid	91.8±3.6 (85.7-98.3)	100.1±7.0 (81.0-103.0)	98.2±5.8 (90.0-107.2)	–
Distance from anterior end to distal end of median bulb	70.0±3.0 (66.4-77.7)	69.4±3.7 (61.4-75.1)	70.0±3.7 (63.5-74.2)	–
Distance from anterior end to pharynx-intestine junction	83.7±3.8 (76.3-91.2)	77.8±4.7 (67.5-84.3)	78.4±3.9 (69.7-81.9)	–
Distance from anterior end to posterior end of pharyngeal glands	140.2±10.9 (126.7-159.8)	126.7±11.0 (108.6-146.9)	134.9±11.0 (117.5-150.8)	–

Table 4.9 (Continued)

Anterior genital branch	301.8±58.0 (246.6-484.5)	492.2±57.2 (382.9-555.9)	371.8±65.1 (293.0-497.1)	–
Posterior genital branch	112.5±10.4 (90.8-125.0)	100.6±13.2 (76.4-117.8)	124.9±14.1 (98.6-146.4)	111.2±10.7 (100.0-120.4)
Body diam. at vulva	19.3±0.7 (18.4-20.9)	21.0±2.0 (17.7-23.2)	21.6±2.3 (19.0-26.8)	–
Vulva to anus distance	153.7±11.8 (138.1-185.6)	165.3±15.5 (144.8-194.5)	177.4±23.8 (148.2-220.4)	–
Distance from anterior end to vulva	535.7±53.8 (445.3-634.9)	616.2±54.0 (510.9-702.8)	637.1±78.3 (498.7-739.7)	–
G1 (%)	41.8±6.5 (36.0-60.7)	44.1±5.4 (37.8-51.1)	43.8±5.2 (37.9-56.1)	–
G2 (%)	15.7±1.7 (11.4-17.5)	12.3±1.2 (11.1-14.0)	14.8±1.1 (13.0-16.6)	–
Anal/cloacal body diam.	9.0±0.5 (8.0-9.8)	9.8±1.0 (8.6-12.0)	9.2±0.3 (8.7-9.7)	–
Tail length	31.7±2.2 (28.6-36.9)	34.5±3.0 (29.6-38.8)	33.5±3.3 (29.5-37.7)	–

4.3.1.2.4 *Bursaphelenchus thailandae*

The morphometrics of males and females of *B. thailandae* isolated from packaging wood imported from Hongkong (Tables 4.10 and 4.11) were compared with those of the type isolate from Thailand (Braasch & Braasch-Bidasak, 2002) and two Chinese isolates from packaging wood imported in Austria (Tomiczek *et al*., 2003; Palmisano *et al*., 2004). The male and female body length of Chinese isolate described in Palmisano *et al.* (2004) (706.6-981.3 μm and 747.8-1194.3 μm, respectively) is longer than that of the Thai type isolate (435.0-720.0 μm and 640.0-820.0 μm, respectively) and my isolate from Hongkong (570.7-832.5 μm and 618.2-840.0 μm, respectively) and that from China in Tomiczek *et al.* (2003) (540.0-880.0 μm and 630.0-840.0 μm, respectively). The a value of males and females of the type population (29.0-46.0 and 36.0-41.0, respectively) is larger than that of the Hongkong and China isolates (26.9-37.0 and 26.0-37.9, respectively). The female c value of the type population (22.0-37.0) is much larger than the Hongkong isolate (11.4-13.2) and the two Chinese isolates intercepted in Austria (12.0-14.0 and 15.1-23.8). The distance from anterior end to distal end of median bulb in original description (male: 37.0-45.0 μm; female: 42.0-50.0 μm) is different from the Hongkong isolate (male: 57.2-66.1 μm; female 57.2-62.9 μm) and the Chinese isolate in Palmisano *et al*. (2004) (male: 69.0-77.4 μm; female: 65.3-84.7 μm).

Table 4.10 Morphometric characters of males of a *Bursaphelenchus thailandae* isolate from packaging wood originating from Hongkong, compared with morphometrics of type population and two other Chinese populations. Measurements in μm and in form: mean standard ± deviation (range).

Characteristic	BtHK	Thailand (Braasch & Braasch-Bidasak, 2002)	China (Tomiczek *et al*., 2003)	China (Palmisano *et al.*, 2004)
n	15	10	15	20
L	658.5±68.0 (570.7-832.5)	582±90 (435-720)	741±100 (540-880)	829.7±71.4 (706.6-981.3)
a	32.0±2.1 (28.5-35.4)	39±5.0 (29-46)	32±2.6 (27-37)	30.7±3.1 (26.9-33.9)
b	9.6±0.7 (8.5-11.3)	7.8±0.9 (6.9-9.3)	7.8±2.4 (6.4-8.8)	10.9±1.0 (9.3-13.1)
b'	4.9±0.3 (4.2-5.6)	–	–	5.5±0.7 (4.3-7.1)
c	24.5±3.6 (20.5-33.9)	28±4.1 (22-37)	27±2.9 (22-32)	26.8±2.6 (20.9-31.2)
c'	2.0±0.2 (1.7-2.3)	1.8±0.2 (1.4-2.1)	–	2.0±0.2 (1.6-2.3)
Lip region diam.	7.7±0.4 (6.8-8.2)	–	–	7.1±0.8 (5.4-7.9)
Lip constriction diam.	7.4±0.4 (6.5-8.0)	–	–	–
Lip region height	3.6±0.3 (3.1-4.0)	–	–	3.7±0.4 (3.0-4.8)
Stylet	15.9±0.5 (15.0-16.7)	13±1.3 (11-15)	15±0.7 (13-16)	13.9±0.5 (13.3-14.5)
Median bulb length	16.7±1.2 (14.7-18.7)	–	–	–
Median bulb diam.	10.8±1.0 (8.6-13.0)	–	–	–
Median bulb length/median bulb diam.	1.6±0.1 (1.4-1.8)	–	–	–
Maximum body width	20.7±2.8 (16.4-26.9)	–	–	27.0±2.8 (20.6-33.9)
Body diam. at middle of median bulb	15.1±1.0 (13.2-16.7)	–	–	–
Distance from anterior end to excretory pore	87.1±6.5 (81.1-103.0)	–	–	116.9±8.4 (99.2-133.1)
Distance from anterior end to Hemizonid	91.1±7.1 (83.9-107.5)	–	–	–
Distance from anterior end to distal end of median bulb	59.8±2.4 (57.2-66.1)	41±4.0 (37-45)	–	72.5±2.2 (69.0-77.4)
Distance from anterior end to pharynx-intestine junction	68.9±3.6 (63.7-77.8)	–	–	76.3±2.4 (72.6-79.9)
Distance from anterior end to posterior end of pharyngeal glands	134.8±15.7 (112.9-174.6)	–	–	–
Anterior genital branch	280.5±28.9 (226.2-346.4)	–	–	583.6±99.3 (423.5-774.4)
Anal/cloacal body diam.	13.4±0.8 (10.8-14.1)	–		–
Tail length	27.1±2.4 (24.1-30.7)	21±3.7 (17-25)	–	31.0±1.6 (29.0-33.9)
T	42.9±5.0 (31.7-53.4)	–	–	70.1±7.7 (59.9-80.3)
Spicule (condylus to distal end)	16.6±1.6 (14.2-18.8)	14±1.6* (12-17)	17±2.0 (13-20)*	17.7±0.5 (16.9-18.2)
Spicule (rostrum to distal end)	8.6±1.1 (6.3-9.7)	–	–	–

Table 4.10 (Continued)

Spicule (curved median line)	12.1±1.5 (9.2-14.8)	–	–	–
Spicule (rostrum to condylus)	8.3±0.9 (6.8-10.0)	–	–	–
Spicule width (measured posterior to rostrum)	2.8±0.3 (2.3-3.4)	–	–	–

* Spicules measured across chord (distal to proximal end), are value =14% more on average.

Table 4.11 Morphometric characters of females of a *Bursaphelenchus thailandae* isolate from packaging wood originating from Hongkong, compared with morphometrics of type population and two other Chinese populations. Measurements in μm and in form: mean standard ± deviation (range).

Characteristic	BtHK	Thailand (Braasch & Braasch-Bidasak, 2002)	China (Tomiczek *et al*., 2003)	China (Palmisano *et al.*, 2004)
n	15	6	10	20
L	672.1±28.7 (618.2-722.3)	727±69 (640-820)	743±65 (630-840)	910.6±148.2 (747.8-1194.3)
a	32.5±2.0 (28.1-36.6)	38±1.9 (36-41)	33±3.7 (26-36)	32.8±2.3 (28.2-37.9)
b	10.0±0.4 (9.5-11.0)	11.0±1.5 (8.7-13.0)	6.2±0.5 (5.5-7.3)	11.7±1.5 (10.0-14.4)
b'	5.0±0.2 (4.7-5.4)	–	–	5.9±1.1 (4.9-8.6)
c	12.2±0.6 (11.4-13.2)	31.±0.6 (22-37)	12±0.6 (12-14)	18.0±2.3 (15.1-23.8)
c'	5.4±0.3 (4.8-6.2)	4.1±1.3 (2.6-5.1)	–	3.8±0.4 (3.0-4.7)
V	72.8±1.6 (68.6-75.1)	73±0.4 (72-73)	73±1.4 (71-75)	76.0±1.1 (73.6-77.9)
Lip region diam.	8.0±0.4 (7.3-8.9)	–	–	8.0±0.6 (7.3-8.5)
Lip constriction diam.	7.6±0.5 (6.8-8.6)	–	–	–
Lip region height	3.6±0.2 (3.2-4.0)	–	–	4.0±0.5 (3.6-4.8)
Stylet	15.7±0.7 (14.8-17.4)	13±0.5 (12-13)	15±0.7 (13-16)	14.0±0.6 (13.3-15.1)
Median bulb length	16.5±0.8 (15.0-17.8)	–	–	–
Median bulb diam.	11.1±1.0 (9.7-12.3)	–	–	–
Median bulb length/median bulb diam.	1.5±0.1 (1.4-1.7)	–	–	–
Maximum body width	20.7±1.7 (18.8-24.2)	–	–	27.8±4.8 (21.8-36.3)
Body diam. at middle of median bulb	15.4±0.7 (14.2-16.6)	–	–	–
Distance from anterior end to excretory pore	85.0±3.5 (79.5-91.5)	–	–	116.1±10.1 (101.6-140.4)
Distance from anterior end to Hemizonid	88.8±3.5 (83.3-96.4)	–	–	–
Distance from anterior end to distal end of median bulb	59.6±1.7 (57.2-62.9)	44±4.2 (42-50)	–	73.6±5.0 (65.3-84.7)
Distance from anterior end to pharynx-intestine junction	67.1±2.8 (63.9-74.4)	–	–	77.6±5.0 (69.0-88.3)
Distance from anterior end to posterior end of pharyngeal glands	133.4±1.7 (122.9-144.6)	–	–	–

Table 4.11 (Continued)

Anterior genital branch	214.0±34.8 (162.9-283.2)	–	–	452.6±153.2 (290.4-776.8)
Posterior genital branch	70.8±7.9 (52.0-85.3)	–	–	84.8±17.8 (60.5-121.0)
Body diam. at vulva	16.9±1.1 (15.6-19.2)	–	–	–
Vulva to anus distance	127.3±10.2 (112.7-154.9)	138±13.1 (120-155)	–	169.6±31.3 (139.2-242.0)
Distance from anterior end to vulva	489.6±27.0 (442.6-538.2)	–	–	–
G1 (%)	31.8±4.9 (25.1-43.5)	–	–	48.7±9.1 (38.5-65.8)
G2 (%)	10.5±1.1 (7.9-12.3)	–	–	9.3±1.0 (7.8-11.7)
Anal/cloacal body diam.	10.3±0.6 (8.7-11.2)	–	–	–
Tail length	55.2±2.5 (51.0-59.1)	59±6.5 (50-65)	–	50.8±6.0 (42.4-72.6)

4.3.1.2.5 *Bursaphelenchus rainulfi*

The morphometrics of the two *B. rainulfi* isolates are shown in Tables 4.12 and 4.13, in which they are compared with data from the Malaysian type isolate (Braasch & Burgermeister, 2002), as well as published data from a Japanese (Wang *et al*., 2005) and a Chinese isolate (Xu *et al*., 2006). The body length of males of all isolates are largely within the range of the type isolate, but the male tail length of the type isolate (20-25 μm) is shorter than that of other isolates (25-33 μm), giving rise to the larger c value (19-38) compared with those of the other isolates (15-26). The distance from anterior end to distal end of the median bulb of the type isolate (male: 41-53 μm; female: 46-53 μm) (Braasch & Burgermeister, 2002) was clearly different from that of the Korean and German isolate (male: 59-66 μm; female 61-70 μm).

Table 4.12 Morphometric characters of males of *Bursaphelenchus rainulfi* isolates from South Korea (BrKOR) and Germany (BrGER), compared with morphometrics of type population and two Chinese populations. Measurements in μm and in form: mean standard ± deviation (range).

Characteristic	BrKOR	BrGER	Malaysia (Braasch & Burgermeister, 2002)	Japan (Wang *et al.,* 2005)	Zhejiang, China (Xu *et al.,* 2006)
n	15	15	15	12	15
L	617.0±27.9 (558.6-650.9)	562.5±27.4 (514-596)	584±74 (475-750)	595±44 (546-668)	523.8±63.7 (429.0-616.0)
a	40.3±2.6 (35.9-45.5)	34.4±2.1 (30.0-37.7)	35±5.0 (25-44)	35±2.2 (32-36))	39.7±4.2 (34.4-45.8)
b	8.4±0.4 (7.6-9.0)	7.8±0.3 (7.3-8.3)	8.0±0.9 (6.8-9.2)	8.7±0.7 (8.0-10.0)	7.9±1.3 (5.6-10.6)
b'	4.9±0.3 (4.3-5.4)	4.6±0.2 (4.0-4.9)	–	–	–

Table 4.12
(Continued)

c	20.2±1.2 (18.7-23.3)	19.0±1.2 (16.9-21.5)	25±4.4 (19-38)	21±3.4 (18-26))	18.8±2.1 (15.1-22.2)
c'	2.8±0.2 (2.5-3.0)	2.6±0.2 (2.4-2.9)	2.5±0.2 (2.2-2.9)	3.0±0.4 (2.5-3.4)	2.5±0.3 (2.0-0.3)
Lip region diam.	5.9±0.3 (5.3-6.2)	5.8±0.3 (5.3-6.4)	–	–	–
Lip constriction diam.	5.1±0.4 (4.4-5.8)	5.2±0.2 (4.8-5.5)	–	–	–
Lip region height	3.2±0.2 (2.8-3.5)	3.1±0.2 (2.8-3.5)	–	–	–
Stylet	13.6±0.5 (13.0-14.7)	13.7±0.3 (13.3-14.2)	12±0.6 (11-13)	13±0.5 (13-14)	12.2±0.9 (10.2-13.9)
Median bulb length	13.7±0.8 (12.5-15.3)	13.7±0.8 (12.9-15.4)	–	–	–
Median bulb diam.	9.7±0.6 (8.7-10.8)	10.0±0.6 (8.8-11.0)	–	–	–
Median bulb length/median bulb diam.	1.4±0.1 (1.3-1.5)	1.4±0.1 (1.2-1.5)	–	–	–
Maximum body width	15.3±0.8 (13.9-16.5)	1.4±1.4 (14.4-19.4)	–	–	–
Body diam. at middle of median bulb	12.7±0.3 (11.9-13.4)	13.1±0.5 (12.3-13.7)	–	–	–
Distance from anterior end to excretory pore	81.6±1.9 (76.5-84.0)	75.1±2.8 (70.6-79.0)	–	–	–
Distance from anterior end to Hemizonid	84.7±2.0 (79.8-87.2)	78.4±2.8 (73.6-81.9)	–	–	–
Distance from anterior end to distal end of median bulb	62.8±1.3 (60.5-65.5)	61.0±1.7 (58.6-64.0)	48±3.4 (41-53)	–	–
Distance from anterior end to pharynx - intestine junction	73.7±1.7 (71.7-77.8)	72.4±2.2 (69.0-75.9)	–	–	–
Distance from anterior end to posterior end of pharyngeal glands	125.8±4.8 (119.4-132.6)	123.0±4.9 (116.5-130.6)	–	–	–
Anterior genital branch	318.6±31.8 (272.0-379.0)	283.5±30.0 (228.7-336.9)	–	–	–
Anal/cloacal body diam.	11.0±0.4 (10.1-11.5)	11.2±0.5 (10.4-11.9)	–	–	–
Tail length	30.5±1.6 (26.6-32.6)	29.6±1.5 (27.3-31.8)	23±0.2 (20-25)	29±4.1 (25-33)	
T	51.8±5.4 (42.6-61.6)	50.3±3.4 (44.5-57.8)	–	–	–
Spicule (condylus to distal end)	12.0±0.8 (10.8-13.1)	12.5±0.7 (11.0-13.2)	13±1.1* (12-15)	13±0.5 (13-14)	11.4±0.7 (10.3-12.6)
Spicule (rostrum to distal end)	6.7±0.6 (5.3-7.6)	6.9±0.3 (6.4-7.6)	–	–	–
Spicule (curved median line)	8.7±0.5 (7.6-9.7)	9.3±0.5 (8.3-10.7)	–	–	–
Spicule (rostrum to condylus)	6.6±0.4 (5.9-7.3)	7.0±0.6 (5.8-8.0)	–	–	–
Spicule width (measured posterior to rostrum)	2.6±0.2 (2.2-2.9)	2.8±0.3 (2.0-3.2)	–	–	–

*Spicules measured across chord (distal to proximal end), are value =11% more on average.

Table 4.13 Morphometric characters of females of *Bursaphelenchus rainulfi* isolates from South Korea (BrKOR) and Germany (BrGER), compared with morphometrics of type population and two Chinese populations. Measurements in μm and in form: mean standard ± deviation (range).

Characteristic	BrKOR	BrGER	Malaysia (Braasch & Burgermeister, 2002)	Japan (Wang *et al.,* 2005)	Zhenjiang, China (Xu *et al.*, 2006)
n	15	15	15	9	15
L	657.2±53.5 (552.8-725.5)	636.6±32.0 (565-682)	661±61 (525-750)	690±37 (655-741)	584.5±64.8 (482-687)
a	38.0±2.3 (35.1-42.4)	34.6±1.8 (31.3-37.7)	32±5.1 (23-40)	35±1.6 (33-37)	34.4±1.1 (33.0-36.6)
b	8.6±0.7 (7.7-10.4)	8.4±0.4 (7.7-9.1)	9.1±0.7 (7.5-10.0)	9.7±1.2 (8.1-10.8)	7.9±1.2 (6.1-9.8)
b'	5.0±0.3 (4.4-5.7)	5.0±0.3 (4.4-5.4)	–	–	–
c	18.0±1.8 (15.2-22.5)	17.4±1.0 (15.1-19.7)	17±1.5 (15-20)	19±0.8 (18-20))	15.6±0.8 (14.7-17.2)
c'	4.5±0.5 (4.0-5.7)	4.3±0.3 (3.6-4.7)	4.2±0.5 (3.4-4.9)	4.0±0.2 (3.5-4.6)	4.0±0.4 (3.5-4.8)
V	73.2±2.1 (67.7-76.2)	72.8±0.8 (70.8-74.5)	74±1.3 (72-76)	74±0.8 (73-75)	72.5±0.6 (71.3-73.8)
Lip region diam.	6.1±0.3 (5.5-6.7)	6.2±0.4 (5.5-6.7)	–	–	–
Lip constriction diam.	5.3±0.3 (4.9-5.8)	5.5±0.4 (4.9-6.3)	–	–	–
Lip region height	3.2±0.2 (2.9-3.6)	3.1±0.3 (2.8-3.7)	–	–	–
Stylet	14.2±0.4 (13.4-15.0)	14.0±1.0 (12.1-15.4)	12±0.8 (11-14)	14±0.5 (14-15)	13.0±0.5 (11.9-13.7)
Median bulb length	14.7±0.7 (13.4-15.7)	14.7±0.8 (13.7-16.6)	–	–	–
Median bulb diam.	10.6±0.7 (9.0-11.5)	11.2±0.8 (10.1-13.2)	–	–	–
Median bulb length/median bulb diam.	1..4±0.7 (1.3-1.6)	1.3±0.1 (1.2-1.4)	–	–	–
Maximum body width	17.3±0.9 (15.3-19.2)	18.4±1.3 (16.4-21.5)	–	–	–
Body diam. at middle of median bulb	14.1±0.7 (12.3-14.9)	14.5±0.7 (13.6-15.9)	–	–	–
Distance from anterior end to excretory pore	83.9±3.2 (78.5-89.2)	78.4±2.8 (74.8-84.3)	–	–	–
Distance from anterior end to Hemizonid	83.9±3.2 (78.5-89.3)	81.8±2.7 (78.3-87.8)	–	–	–
Distance from anterior end to distal end of median bulb	64.1±2.4 (60.6-69.8)	63.5±1.5 (60.7-66.7)	48±2.7 (46-53)	–	–
Distance from anterior end to pharynx-intestine junction	74.3±3.3 (69.7-80.7)	75.6±3.0 (71.7-83.9)	–	–	–
Distance from anterior end to posterior end of pharyngeal glands	130.8±7.3 (122.4-145.3)	127.9±4.4 (124.0-137.9)	–	–	–
Anterior genital branch	260.8±37.7 (187.0-322.6)	258.6±25.5 (228-306)	–	–	–
Posterior genital branch	65.6±8.2 (55.2-80.5)	64.1±6.8 (52.3-79.5)	–	–	64.5±12.2 (53.4-83.8)
Body diam. at vulva	15.7±0.3 (4.4-5.7)	16.6±0.8 (15.5-18.0)		–	–

Table 4.13 (Continued)

Vulva to anus distance	138.5±8.7 (124.3-150.4)	136.2±7.4 (121.3-148.6)	135±10.6 (120-155)	143±0.2 (132-154)	–
Distance from anterior end to vulva	482.1±49.4 (394.5-537.5)	463.8±25.4 (409.9-508.3)	–	–	–
G1 (%)	40.0±3.6 (33.8-45.0)	40.7±4.1 (33.9-49.2)	–	–	–
G2 (%)	10.0±1.4 (8.0-12.8)	10.1±1.3 (8.1-12.6)	–	–	–
Anal/cloacal body diam.	8.1±0.4 (7.3-8.7)	8.5±0.4 (7.7-9.1)	–	–	–
Tail length	36.6±3.0 (31.6-41.5)	36.6±2.6 (31.6-41.9)	38±3.4 (32-45)	35±2.1 (33-38)	–

The CDA analysis of the morphometrics of all isolates the *Bursaphelenchus* species isolated used nine characters for males and eight characters for females as variates (Table 4.14).

Table 4.14 Standardised coefficients for canonical variates for males and females of 15 isolates of five described *Bursaphelenchus* species.

	Males		Females	
	Root 1	Root 2	Root 1	Root 2
% of variation	80.67	12.33	81.89	9.72
Selected characters	Vector Loadings		Vector Loadings	
Body length	0.0099	-0.0115	-0.0022	-0.0083
Stylet length	0.1599	0.5839	0.2193	0.4221
a	-0.1119	-0.1778	-0.2310	-0.3515
b'	0.3650	-0.0130	0.4975	0.9841
c	-0.1005	0.0430	-0.2192	0.1946
c'			1.4740	-0.0220
V			0.0901	0.2632
Post-uterine sac length			-0.0220	0.0179
spicule length	-0.4595	-0.3617		
Spicule rostrum to distal end	-0.5238	0.3218		
Spicule rostrum to condylus	-0.0832	0.0150		
Spicule condylus to rostrum	0.1085	0.4208		

The CDA clearly separated the five *Bursaphelenchus* species by male characters (Fig 4. 7A) and female characters (Fig 4.7B). Three morphometrical traits of males (spicule rostrum to distal end, spicule length and b' value) provided the most useful taxonomic characters. For the female, the values c', b' and a, were the best characters for species separation.

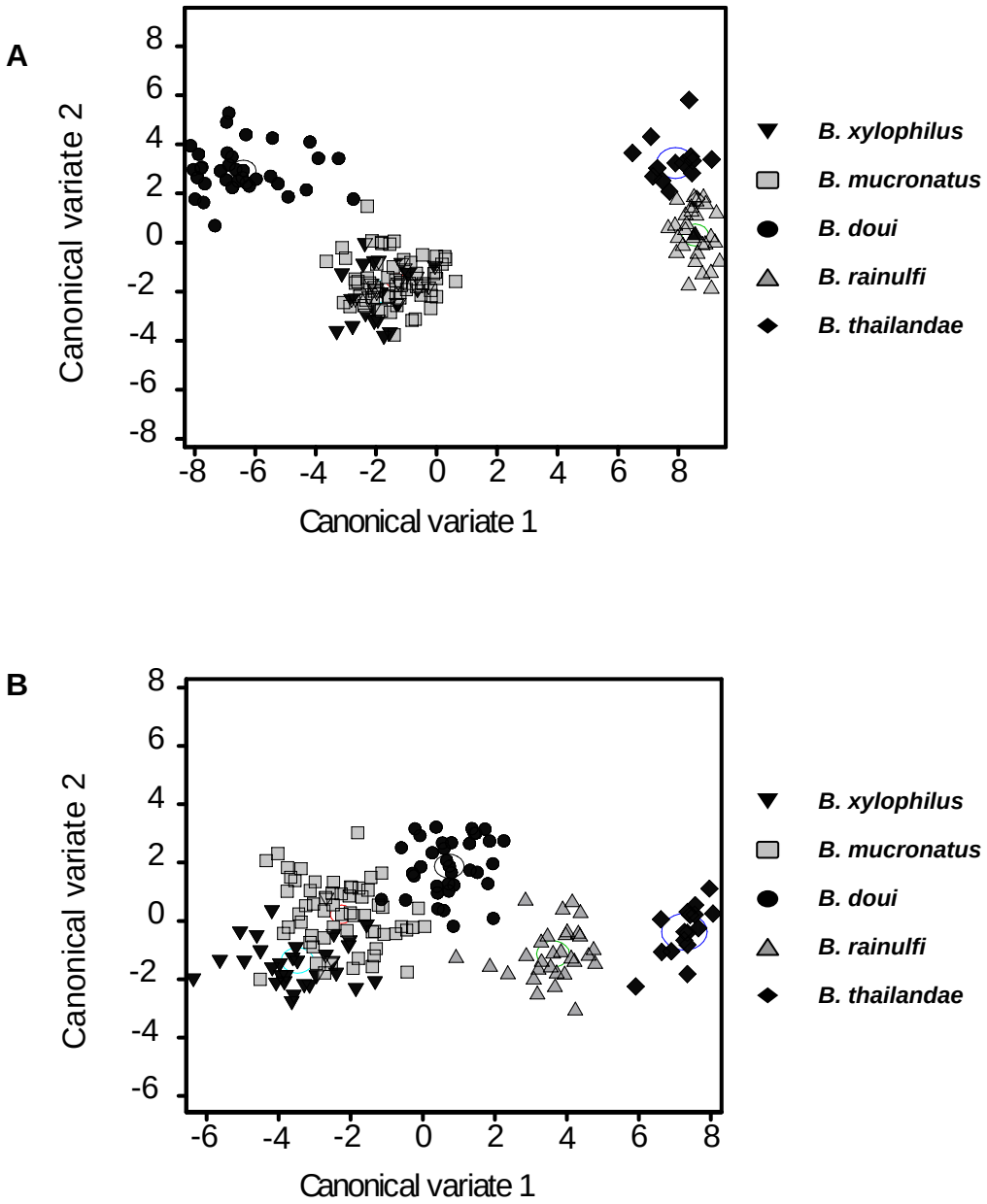

Fig 4.7 Canonical discriminant analysis of morphometric characteristics of 15 isolates of five described *Bursaphelenchus* spp. for male (A) and female (B) performed with nine and eight variables respectively (Table 4.14). The circles display 95% confidence regions.

4.3.1.3 Molecular characterisation

4.3.1.3.1 ITS-RFLP patterns

When compared with published ITS-RFLP patterns (Braasch & Burgermeister, 2002; Braasch *et al.,* 2004a; Burgermeister *et al*., 2005b), the ITS-RFLP patterns obtained for described *Bursaphelenchus* species isolated from packaging wood in Nanjing confirmed their morphological and morphometrical identification (Fig 4.8).

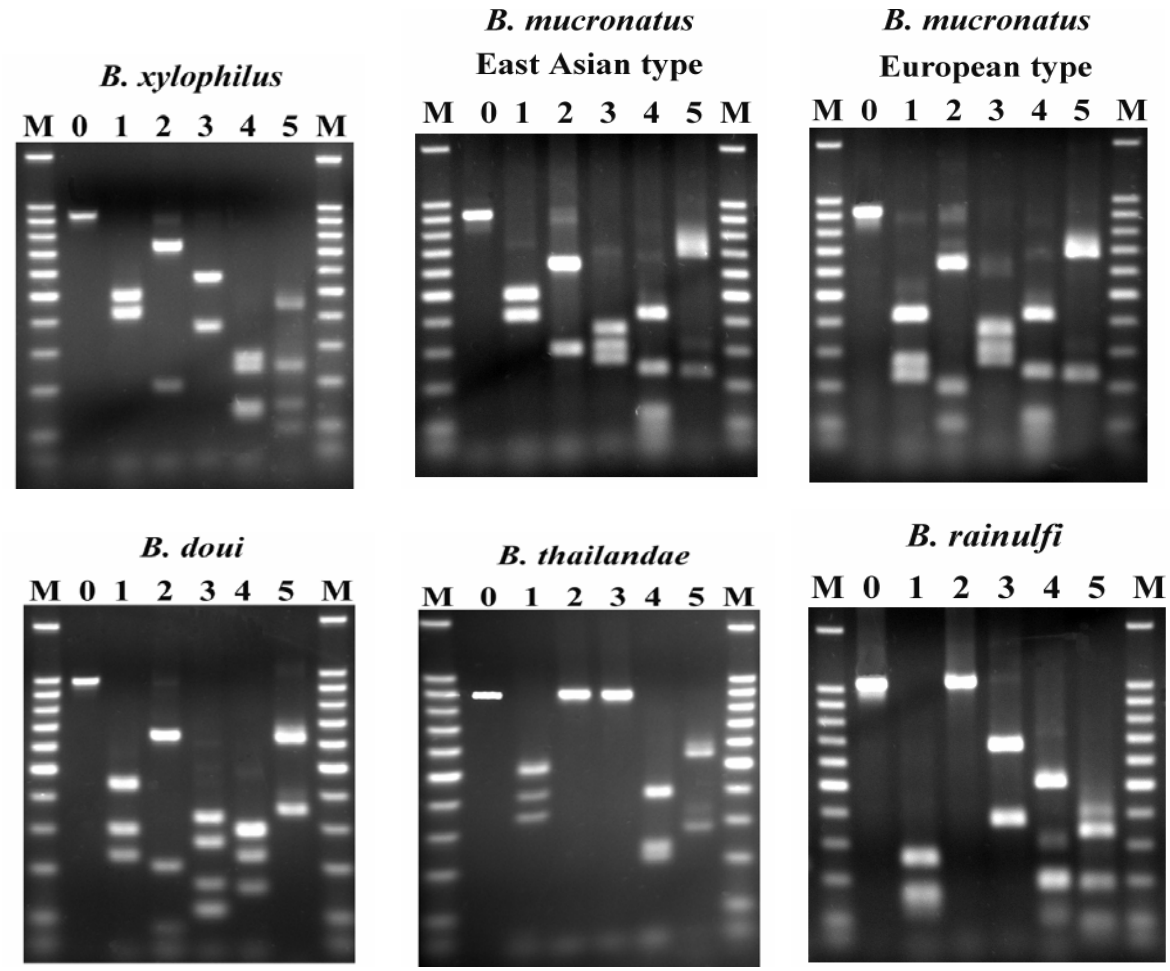

Fig 4.8 ITS-RFLP patterns of *B. xylophilus* (BxCAN), *B. mucronatus* East Asian type (BmCAN), *B. mucronatus* European type (BmMEX), *B. doui* (BdKOR), *B. rainulfi* (BrGER) and *B. thailandae* (BtHK).Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with *Rsa*I (1), *Hae*III (2). *Msp*I (3), *Hinf*I (4) and *Alu*I (5). M: DNA marker (100bp ladder, Promega).

The two morphological forms of *B. xylophilus*, *viz.* the R-form (BxJAP) and the M-form (BxCAN) showed identical ITS-RFLP patterns (Fig 4.8). The five *B. mucronatus* isolates split into two groups on the basis of their ITS-RFLP patterns differing in the number and size

of rDNA restriction fragments obtained upon digestion with *Rsa*I and *Hae*III (Hoyer *et al*., 1998). The isolates from Germany, Mexico and Sweden were molecularly classified as European types; those from Canada and Hongkong were identified as East Asian types. The ITS-RFLP patterns of both *B. doui* and *B. thailandae* were identical to those published by Braasch *et al.* (2004a) and Burgermeister *et al.* (2005b). The ITS-RFLP pattern obtained for *B. rainulfi* was identical to the pattern in Braasch and Burgermeister (2002).

The size of restriction fragments of amplified ITS-rDNA from five described *Bursaphelenchus* species are listed in Table 4.15.

Table 4.15 Restriction fragments of amplified ITS-rDNA from five described *Bursaphelenchus* species originating from packaging wood of different origin.

Bursaphelenchus Species	ITS-PCR product (bp)	Restriction fragments (bp)				
		*Rsa*I	*Hae*III	*Msp*I	*Hinf*I	*Alu*I
B. xylophilus	950	500	730	570	270	460
		420	200	380	260	250
					140	140
						100
B. mucronatus (East Asian type)	950	500	620	370	410	700
		410	310	310	250	250
				280	130	
					90	
B. mucronatus (European type)	950	410	620	370	410	700
		290	220	310	250	250
		230	110	280	130	
					90	
B. doui	1000	450	650	330	300	620
		310	210	280	240	360
		240	80	170	170	
				120		
B. rainulfi	1050	270	1050	690	510	340
		180		390	210	200
		160			200	100
		120			110	
B. thailandae	900	500	900	900	400	540
		400			230	290
		180			210	

4.3.1.3.2 Phylogeny

The mean intraspecific pairwise sequence divergence of the D2D3 region of each of the five described *Bursaphelenchus* species ranged from 0.2 to 0.4%. The divergence between the species of *xylophilus* group (*B. xylophilus, B. mucronatus and B. doui*) ranged from 1.4 to 5.9%. The sequence divergences between the *B. xylophilus* group and *B. rainulfi* and *B. thailandae* ranged from 21.9 to 23.1% and from 26.1 to 27.1%, respectively.

Phylogenetic trees were generated by bootstrapping tests of minimum evolution (ME) and equally weighted maximum parsimony (MP) in PAUP (4.0) (Fig 4.9A-B). The phylogenetic analysis supported the identification of *Bursaphelenchus* species obtained from the morphological and morphometric studies. The 100% of bootstrap values demonstrated BrKOR and BrGER from South Korea and Germany respectively grouped with *B. rainulfi.* BtHK from Hongkong stayed with *B. thailandae.* BdKOR and BdJAP from South Korea and Japan respectively grouped with *B. doui* with 100% bootstrap value and joined into *xylophilus*-group with the rest of *Bursaphelenchus* isolates, three are *B. xylophilus* and six are *B. mucronatus* which included two subgroup, East-Asian type and European type.

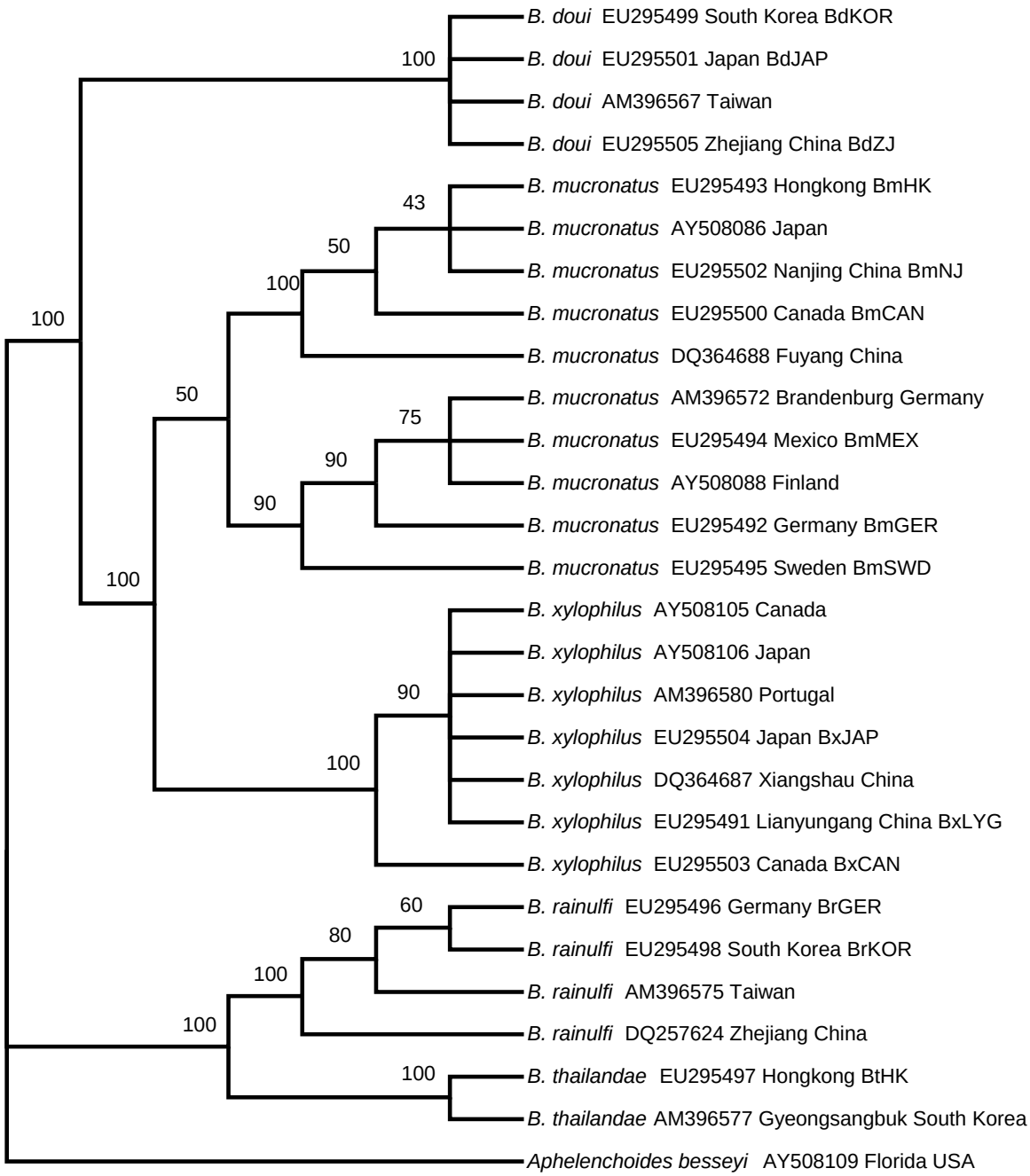

Fig 4.9A Minimum Evolution tree (60% consensus) of described *Bursaphelenchus* species isolated from packaging wood with addition of some populations of the same species found in Genbank. *Aphelenchoides besseyi* is used as outgroup. Bootstrap values (%) are given for each node.

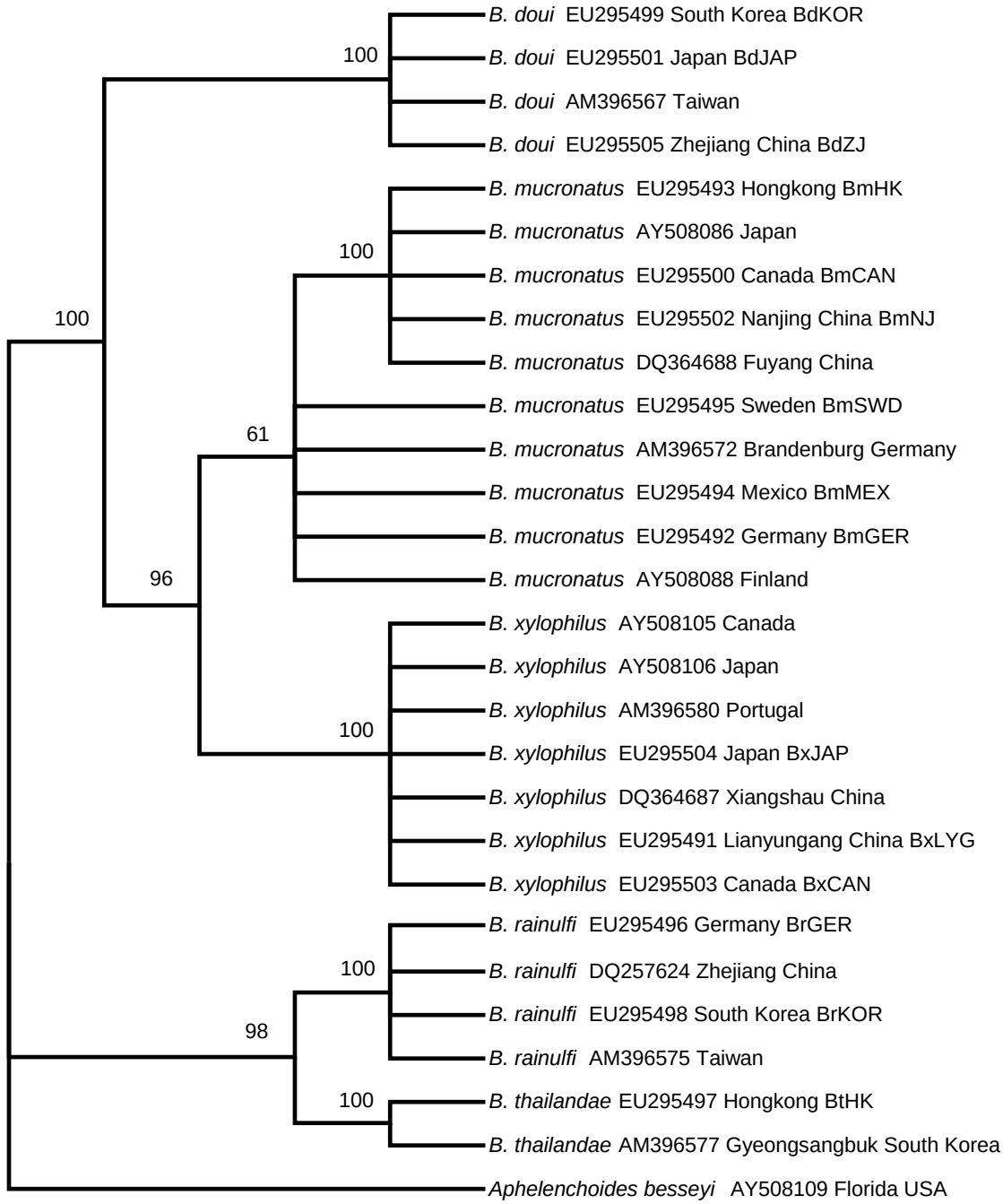

Fig 4.9B Maximum Parsimony tree (60% consensus) of described *Bursaphelenchus* species isolated from packaging wood with addition of some populations of the same species found in Genbank. *Aphelenchoides besseyi* is used as outgroup. Bootstrap values (%) are given for each node.

4.3.2 Characteristics of new species, *Bursaphelenchus chengi* Li, Trinh, Waeyenberge & Moens, 2008

4.3.2.1 Measurements

Males and females are listed in Table 4.16.

Table 4.16 Morphometrics of *Bursaphelenchus chengi.* All measurements are in μm and in the form: mean ± standard deviation (range).

	Male		Female
	Holotype	Paratypes	Paratypes
n	-	20	20
L	708.9	722.8±51.0 (645.6-824.8)	741.9±45.5 (661.1-828.0)
a	34.3	29.8±2.0 (25.4-34.3)	30.0±2.0 (25.1-33.9)
b	7.6	8.1±0.8 (6.7-9.5)	9.0±1.0 (7.3-10.8)
b'	5.6	5.2±0.8 (3.3-6.4)	5.8±0.9 (4.2-5.8)
c	17.5	18.4±1.7 (14.8-21.7)	14.8±1.3 (12.7-17.2)
c'	3.1	2.6±0.3 (2.2-3.2)	4.4±0.4 (3.6-5.1)
V	–	–	73.2±2.0 (69.5-77.0)
Lip region diam.	7.3	7.66±0.5 (6.9-8.7)	7.7±0.4 (6.8-8.5)
Lip constriction diam.	6.8	6.8±0.4 (6.0-7.5)	7.0±0.3 (6.5-7.6)
Lip region height	3.3	3.6±0.3 (3.2-4.6)	3.6±0.2 (3.2-4.0)
Stylet length	15.5	15.8±0.9 (14.1-16.9)	15.6±1.4 (10.3-17.1)
Median bulb length	17.8	17.7±0.7 (16.3-18.9)	18.2±0.7 (16.8-19.9)
Median bulb diam.	11.6	13.1±0.8 (11.6-14.6)	13.7±0.9 (12.2-15.6)
Median bulb length/median bulb diam.	1.5	1.4±0.1 (1.2-1.5)	1.3±0.1 (1.2-1.5)
Maximum body width	20.7	24.3±1.7 (20.7-27.0)	24.8±1.7 (21.6-28.5)
Body diam. at middle of median bulb	15.5	17.3±1.0 (15.5-19.6)	17.6±0.8 (16.4-19.1)
Distance from anterior end to excretory pore	95.1	93.4±6.1 (80.5-102.7)	90.6±4.1 (84.1-97.3)
Distance from anterior end to Hemizonid	98.8	98.1±6.5 (84.5-109.9)	95.5±4.1 (87.8-103.5)
Distance from anterior end to distal end of median bulb	71.8	70.7±4.0 (63.9-81.9)	66.7±2.8 (62.4-71.2)
Distance from anterior end to pharingo-intestine junction	93.4	89.4±6.7 (72.3-101.7)	83.3±4.7 (75.1-95.0)
Distance from anterior end to posterior end of pharyngeal glands	126.3	142.3±21.5 (118.1-201.6)	130.3±20.3 (84.3-175.6)

Table 4.16 (Continued)

Anterior genital branch	375.2	347.9±46.7 (287.4-478.4)	226.7±31.6 (182.9-285.2)
Posterior genital branch	–	–	93.2±15.4 (58.7-122.5)
Body diam. at vulva	–	–	21.9±1.1 (20.0-23.6)
Vulva to anus distance	–	–	147.7±13.0 (114.4-167.1)
Distance from anterior end to vulva	–	–	543.7±44.2 (463.6-637.9)
G1 (%)	–	–	30.6±4.4 (22.3-38.8)
G2 (%)	–	–	12.6±2.0 (8.6-17.3)
Anal/cloacal body diam.	13.3	15.1±1.0 (13.3-16.9)	11.5±0.8 (10.5-13.0)
Tail length	40.6	39.4±2.7 (35.8-43.9)	50.4±2.8 (45.8-54.8)
T	52.9	48.3±6.8 (36.5-63.0)	–
Spicule (condylus to distal end)	21.3	22.4±1.0 (20.4-24.1)	–
Spicule (rostrum to distal end)	10.5	11.4±0.7 (10.1-12.7)	–
Spicule (curved median line)	17.7	18.0±1.2 (16.0-20.3)	–
Spicule (rostrum to condylus)	11.8	12.5±0.9 (10.4-14.3)	–
Spicule width	5.1	5.6±0.5 (4.8-6.6)	–

4.3.2.2 Morphology

MALE

Displaying all the features of Aphelenchoidoidea according to Hunt (1993). Body slender, cylindrical. Distal part of body curved and J-shaped when killed by gentle heat. Cuticle with fine annulations. Lateral field with two distinct incisures (*i.e.*, with a single ridge or band). Lip region high, rounded, offset by constriction, labial annules lacking. SEM *en face* view of head showing six equal, closely arranged, lips. Oral disc distinct, oral aperture circular, surrounded by six inner papillae. Amphidial apertures pore-like, cephalic papillae obscure. Stylet long, basal knobs absent, but very slight basal swellings present. Median bulb elongate-oval. Secretory/excretory pore located 1.0-1.5 body diam. posterior to nerve ring. Hemizonid located 5-6 μm posterior to excretory pore. Pharyngeal glands overlapping intestine for 2-3 body diam., mostly on dorsal side. Testis monorchic, usually anteriorly outstretched, occasionally reflexed, occupying *ca* one-third to half of body length; cells initially arranged in single row and then in two rows. Spicules paired, robust, rosethorn-shaped, strongly curved; rostrum prominent, not sharply pointed, condylus high,

rounded and well developed; distal end truncate with broad and blunt cucullus offset slightly more from dorsal limb than ventral (SEM). Distal third of dorsal limb laterally expanded forming flattened wing-like alae. Tail arcuate, terminus pointed; bursa usually truncate with posterior margin indented in some specimens or more rounded to a fine, more or less axial, point. Copulatory papillae comprising single precloacal ventromedian papilla, one pair of precloacal subventral papillae at same level, one pair of post cloacal subventral papillae at middle of tail; gland openings appearing as two pairs of small papillae.

FEMALE

Body slightly curved ventral when heat-relaxed. Genital tract monoprodelphic, outstretched, cells initially arranged in single row, thereafter in two rows. Spermatheca differentiated, roundish, irregular rectangular, filled with rounded sperm. Quadricolumella visible. Postuterine branch extending for *ca* 60% of vulva-anus distance, often containing sperm. Vulva inclined anteriad at *ca* 45◦ to body axis. Vulva with anterior lip slightly extended to form a small, but distinct, flap. Tail medium length, conoid, gradually tapering to bluntly rounded or acute terminus with thickened cuticle forming a hyaline region, terminal region bearing irregular, flap-like, cuticular folds (SEM), sometimes with a finely pointed or irregular mucron.

JUVENILE STAGES

Juveniles with conical tail, that of J3 and J4 (female) tail slightly ventrally curved and J4 male tail also ventrally curved. Developing gonad visible in posterior region of J4 male.

4.3.2.3 Type host and locality

Isolated from packaging wood arriving at Nanjing (China) from Taiwan with other commodities and cultured on a lawn of *Botrytis cinerea* growing on PDA.

4.3.2.4 Type material

Holotype male (UGMD104113), ten male paratypes (6 in UGMD 104112 and 4 in UGMD104113) and ten male paratypes (UGMD 104111) deposited in the nematode collection of the Institute of Zoology, Ghent University, K.L. Ledeganckstraat 35, Ghent, Belgium. Six female paratypes and five male paratypes also deposited in the nematode collection of Nanjing Agricultural University, China.

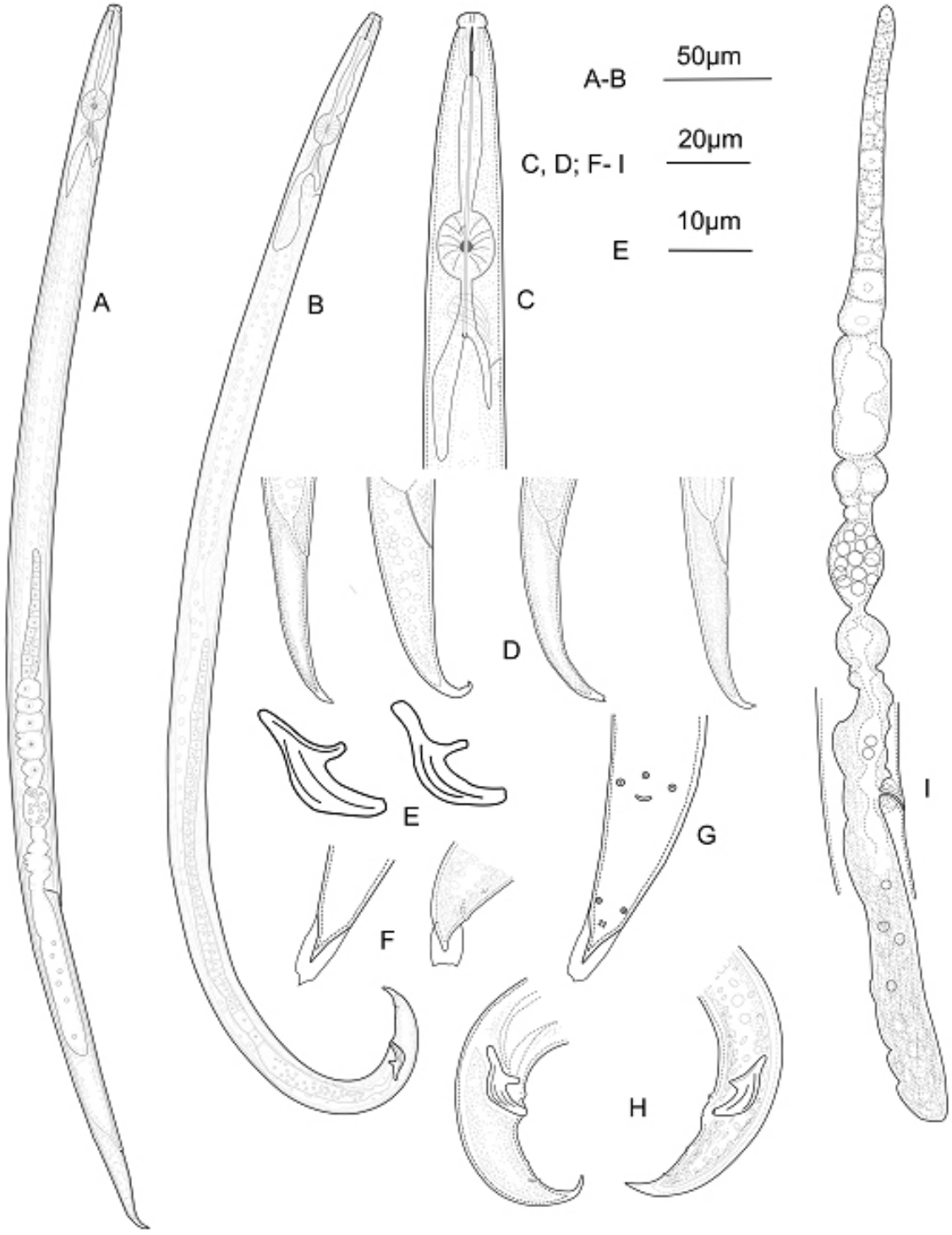

Fig 4.10 *Bursaphelenchus chengi.* A: Entire body of female; B: Entire body of male; C: Female anterior region; D: Female tails; E: Spicule shapes; F: Bursa of males; G: Ventral view of male tail and position of papillae; H: Lateral view of male tails; I: Lateral view of female vulva region and reproductive tract.

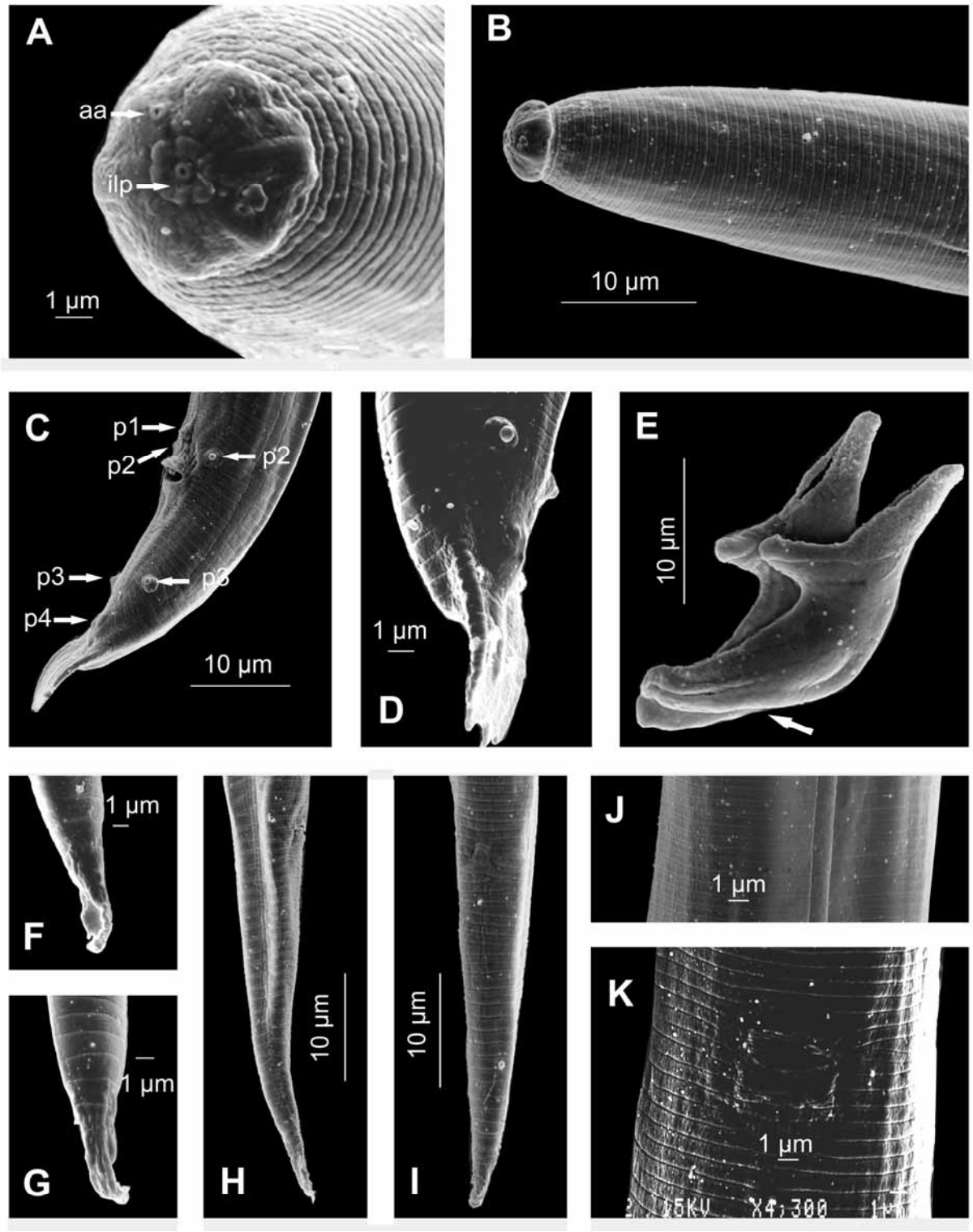

Fig 4.11 Scanning electronic microscopy of *Bursaphelenchus chengi.* A: Enface view of male; aa: amphidial aperture; ilp: inner labial papilla; B: Male head lateral view; C: Male tail lateral view; p1: single precloacal papilla; p2: precloacal subventral papillae; p3: postcloacal subventral papillae; p4: two gland papillae; D: Male tail tip ventral view; E: Spicules (the arrow shows the flattened wing-like structure in the distal third of the dorsal limb); F, G: Variation in tail tip of females; H, I: Variation in female tails; J: Lateral field in middle body of female; K: Vulva in ventral view.

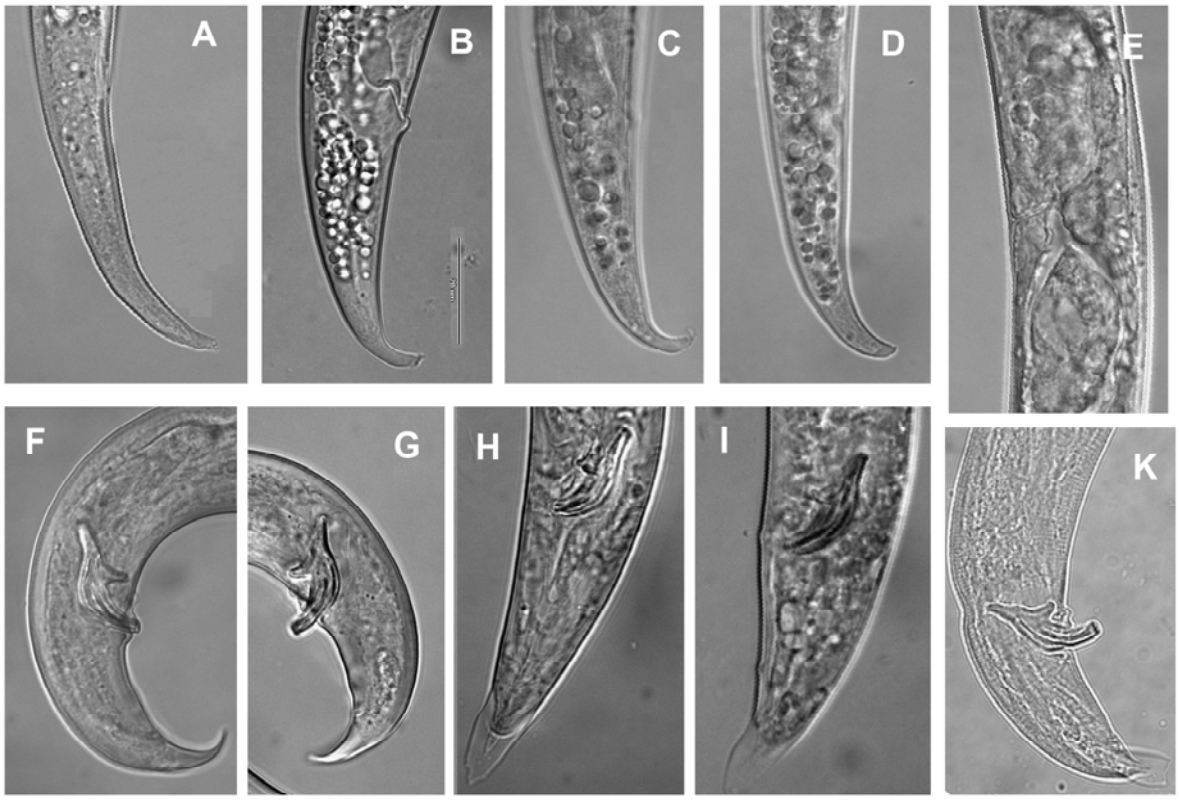

Fig 4.12 Light microscope observation of *Bursaphelenchus chengi*. A-C: Variation in female tails; D: Tail of J4 (female); E: Vulval region; F-G: Male tails; H-K: Bursa in tail of males.

4.3.2.4 Diagnosis and relationships

Bursaphelenchus chengi is characterised by the medium body size in both sexes, the presence of only two incisures in the lateral field and the robust and strongly curved spicules. The spicule lamina is angular distally, the rostrum digitate and the condylus rounded. The tail is arcuate with a pointed terminus. The bursa is usually truncate with the posterior margin indented in some specimens or rounded with a fine terminal point. Females have a small vulval flap formed by a short extension of the cuticle of the anterior lip, and a conical tail that gradually tapers to an almost straight or slightly recurved, pointed or rounded terminus.

Braasch (2001) grouped the species of *Bursaphelenchus* on the basis of the number of lateral lines. Ryss *et al*. (2005), however, classified the species into six groups mainly based on the shape of the spicules. Based on this latter classification, *B. chengi* would be classified within the *piniperdae*-group, *i.e*., a group of species with stout spicules, concave capitulum, elongated condylus, lamina smoothly curved or angular at midpoint, cucullus usually absent although small cucullus sometimes present. However, the *piniperdae*-group *sensu* Ryss *et al*. is an artificial conglomerate of species, members of which may have two, three or four lateral lines and various arrangements of caudal papillae.

Recent studies on the phylogeny of the genus have used a combination of molecular data

with morphological characters, including the number of lateral lines and spicule shape (Lange *et al.*, 2007; Ye *et al.*, 2007). In these recent classifications *B. chengi* clusters in the *abietinus*-group (*B. abietinus* Braasch & Schmutzenhofer, 2000, *B. antoniae* Peñas, Metge, Mota & Valadas, 2006, *B. hellenicus* Skarmoutsos, Braasch & Michalopoulou, 1998, *B. hylobianum* and *B. rainulfi*). The species in this group have two lateral lines, a similar spicule shape, a tapering female tail and the presence of a small vulval flap. The new species can be easily differentiated from the *xylophilus*-group and the *sexdentati*-group (*sensu* Braasch, 2001), both of which have four lateral lines, and from the *leoni*-group which has three lateral lines.

Bursaphelenchus chengi differs from *B. abietinus* by larger and stouter spicules (20-24 *vs* 11-14 μm), spicules lacking a well defined cucullus *vs* with distinct cucullus, one precloacal pair and a single ventromedian precloacal caudal papilla *vs* two precloacal pairs, and a longer male stylet (14-17 *vs* 11-13 μm).

Bursaphelenchus chengi differs from *B. antoniae* by a longer male body (646-825 *vs* 476-660 μm), a broader male lip region (diam. 6.9-8.7 *vs* 5.5-6.0 μm) and a longer male stylet (14-17 *vs* 11-14 μm). *Bursaphelenchus chengi* also has longer spicules (as measured between the condylus and the distal end) of 20-24 *vs* 15- 20 μm, and a longer distance from the distal tip of the rostrum to the condylus (10-14 *vs* 6.5-10 μm). The a value is also different, being 25.4-34.3 *vs* 34.2-44.0. The spicules of *B. chengi* resemble those of *B. antoniae* in shape but are broadly truncate and lack a well defined, disc-like cucullus.

Bursaphelenchus chengi differs from *B. hellenicus* by the truncate distal end of the spicule which bears a broad and blunt cucullus *vs* a distinct cucullus in *B. hellenicus*, the c value of 18.4 (14.8-21.7) *vs* 24 (19-30) in males and 14.8 (12.7-17.2) *vs* 21 (17-31) in females, and the lateral field with two distinct incisures *vs* three (occasionally two) incisures.

The spicules of *B. chengi* resemble those of *B. hylobianum* in shape but have a shorter condylus. *Bursaphelenchus hylobianum* was described as having a cucullus by Braasch and Braasch-Bidasak (2002) although the original description (Korenchenko, 1980) did not mention this feature. The new species also differs from *B. hylobianum* by having three postcloacal papillae pairs of papillae *vs* only one postcloacal pair according to Braasch and Braasch-Bidasak (2002), although Korenchenko (1980), in the original description, reported three pairs to be present.

Bursaphelenchus chengi differs from *B. rainulfi* by longer spicules (22 *vs* 13 μm), a longer male stylet length (15.8 *vs* 12 μm) and the excretory/secretory pore located posterior to the nerve ring *vs* at median bulb level.

4.3.2.5 ITS-RFLP profile

The ITS-RFLP patterns of *B. chengi* (Table 4.17; Fig 4.13) are different from those of the closely related *B. antoniae* (Peñas *et al*., 2006) and *B. hylobianum* (Braasch & Burgermeister, 2002) except for the pattern obtained after *Hae*III digestion. The ITS-RFLP patterns of the new species are also different from those of *B. abietinus*, *B. hellenicus* and *B. rainulfi* (Braasch & Burgermeister, 2002).

Table 4.17 Restriction fragments of amplified ITS-rDNA from *Bursaphelenchus chengi* and five morphologically similar *Bursaphelenchus* species.

Bursaphelenchus species	ITS-PCR product (bp)	Restriction fragments (bp)					
		Rsa I	*Hae*III	*Msp* I	*Hinf* I	*Alu* I	References
B. chengi	1150	610	790	460	340	810	Li *et al*., 2008
		280	360	370	290	280	
		160		310	210		
		90			200		
					110		
B. antoniae	1150	610	790	490	340	790	Peñas *et al.*, 2006
		290	360	370	290	340	
		230			250		
					220		
B. hylobianum	1150	590	790	360	490	1150	Braasch & Burgermeister, 2002
		280	360	310	270		
				180	240		
					120		
B. abietinus	1070	610	670	530	530	580	Braasch & Burgermeister, 2002
		280	230	400	220	230	
		180	170	160	120	50	
B. rainulfi	1050	270	1050	690	510	340	Braasch & Burgermeister, 2002
		180		390	210	200	
		160			200	100	
		120			110		
B. hellenicus	1080	610	530	690	520	340	Braasch & Burgermeister, 2002
		290	390	390	320	280	
		180	160		220	180	
						120	

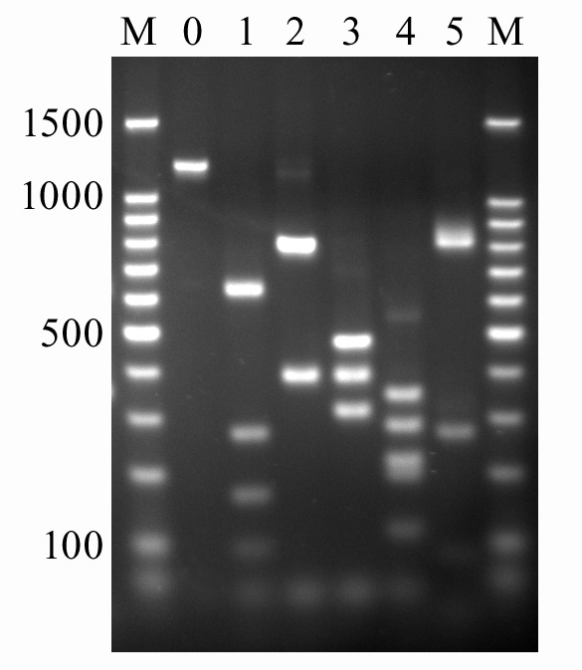

Fig 4.13 ITS-RFLP pattern of *Bursaphelenchus chengi.* Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with *Rsa*I (1), *Hae*III (2). *Msp*I (3), *Hinf*I (4) and *Alu*I (5). M: DNA marker (100bp ladder).

4.3.2.6 Sequence analysis

The sequence length of the 28S rDNA D2/D3 domain of *B. chengi* is 741 bp (GenBank accession no. EU107359). The comparison of the pairwise sequence similarities between *B. chengi* and other related species revealed that the sequence of the new species has the highest similarity value (93.5%) with *B. antoniae* (accession no. AM279710). The sequence similarities between the new species and *B. hylobianum* (AY508085), *B. abietinus* (AY508074), *B. hellenicus* (AY508083) and *B. rainulfi* (DQ257624) are 85.7, 82.3, 80.9 and 76.3%, respectively. *Bursaphelenchus chengi* has only 75.5 and 75.0% similarity with *B. xylophilus* (DQ364687) and *B. mucronatus* (DQ364688), respectively. In the phylogenetic trees *B. chengi* clusters within the *abietinus*-group and is the closest species to *B. antoniae* (Fig 4.14). The phylogenetic analysis supports the conclusion that *B. chengi* is a new species close to *B. antoniae* and other two-lined *Bursaphelenchus* species.

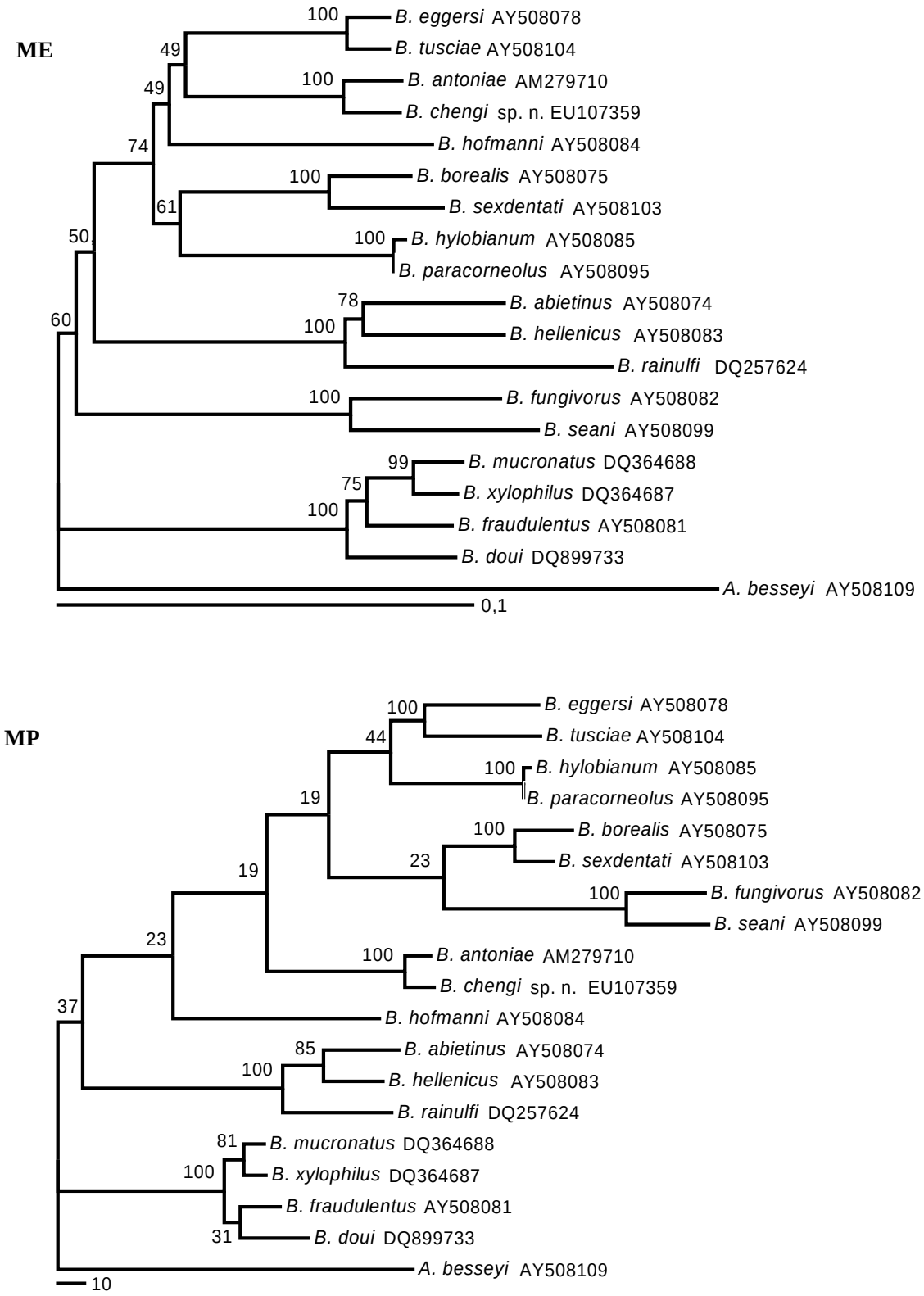

Fig 4.14 Phylogenetic relationships of new species *Bursaphelenchus chengi* and 17 *Bursaphelenchus* species. *Aphelenchoides besseyi* is the outgroup. The global sequence alignments for tree constructions were calculated for 28S D2/D3 domain sequences by minimum evolution (ME) and maximum parsimony (MP) algorithms. Bootstrap values (%) are given for each node.

4.4 Discussion

To inhibit the introduction of PWN, China has implemented in 2000 quarantine regulations for coniferous packing wood imported from North America, Japan, Korea and the European Union. My participation in the inspection of wood packaging materials imported at Nanjing Port during 2003-2004 yielded five described *Bursaphelenchus* species (*viz. B. xylophilus*, *B. mucronatus* (East Asian and European types)*, B. doui*, *B. rainulfi* and *B. thailandae*) and one new species, *B. chengi*. These findings clearly confirm the threat posed by imported packaging wood.

In China, *B. xylophilus* was first intercepted in Nanjing in 1992 when coniferous wood packaging Japanese equipment was sampled (Xu *et al*., 1995). Since then, the nematode has been detected many times in large numbers in wood packaging imported from disease infected countries, e.g. Japan (Ma & Zhang, 1997) and Portugal (Li *et al*., 2003). Recent inspection reports highlight that nearly 20% (40 out of 202) of the batches detected with nematodes contained *B. xylophilus* (Gu *et al*., 2006a). The nematode was also detected in shipments to other regions, e.g. pine wood chips imported from the United States and Canada into Finland (Rautapää, 1986), and coniferous timber imported from the Asian part of Russia to Europe (Braasch *et al.*, 2001). In the inspection carried out in Nanjing, both the M-form (origin Canada) and R-form of *B. xylophilus* (origin Japan) were detected in imported packaging wood. Obviously, introducing *B. xylophilus* continues to be a serious threat.

Bursaphelenchus mucronatus is probably the most frequently detected species in packaging wood. In a survey conducted by Braasch *et al*. (2001) on coniferous wood imported from the Asian part of Russia to Europe, *B. mucronatus* was the predominant species (41 out of 51 batches). The same species was detected in 46% (11 out of 24) of the nematode samples taken from packaging wood imported from China to Austria (Tomiczek *et al.*, 2003). In China, it was isolated from 57 out of 202 batches detected with *Bursaphelenchus* spp. (Gu *et al*., 2006a). I obtained five isolates of *B. mucronatus*; three of them belonged to the European genotype (isolates from Germany, Mexico and Germany) and two matched the East Asian genotype (from Hongkong and Canada). My findings along with other reports clearly indicate that *B. mucronatus* is widespread in several continents.

Bursaphelenchus doui was described from China when it was isolated from imported packaging wood originating from Taiwan and South Korea (Braasch *et al*., 2004a). Later, this species was intercepted four times in packaging wood imported from South Korea and Taiwan (Gu *et al*., 2006a). I found the species in packaging wood imported from Japan and

South Korea and in dead pine tree in China. *Bursaphelenchus doui* might be a species originating in East Asian countries.

Bursaphelenchus rainulfi and *B. thailandae* were first described from *Pinus caribaea* (Braasch & Burgermeister, 2002) and *P. merkusi* trees (Braasch & Braasch-Bidasak, 2002) in Malaysia and Thailand, respectively. In China *B. rainulfi* was first detected in Japanese packaging wood (Wang *et al*., 2005) and in dead *P. massoniana* in Zhejiang Province (Xu *et al*., 2006). Later it was discovered several times in packaging wood imported from Taiwan, South Korea, Japan and Hongkong, Germany and USA (Gu *et al*., 2006a). I isolated the species twice (Germany and South Korea). *Bursaphelenchus thailandae* was earlier discovered in China in wood packaging imported from South Korea, Hongkong, Japan, Italy and USA (Gu *et al*., 2006a). The species was discovered twice in Austria from Chinese packaging wood (Tomiczek *et al*., 2003; Palmisano *et al.*, 2004). I isolated the nematode in packaging wood from Hongkong. The fact that both species are frequently intercepted in packaging wood from East Asian countries illustrates their wide distribution in East Asia. The occasional finding of this species in wood packaging from North American and European countries might be attributed to the circulation of contaminated wood material between different countries by international trade (Gu *et al*., 2006a).

Bursaphelenchus chengi is a new species described and illustrated in this chapter. I isolated the dauer juveniles from imported wood packaging materials from Taiwan to Nanjing Port, China. The similarity of morphological characters showed that the new species is grouped within the *abietinus*-group *sensu* Braasch together with *B. abietinus*, *B. antoniae*, *B. hellenicus*, *B. hylobianum* and *B. rainulfi*. The ITS-RFLP profiles and phylogenetic analysis of the 28S rDNA D2/D3 domain sequence support the new species *B. chengi*.

In view of the quarantine regulations prevailing in several countries or regions, the correct identification of *Bursaphelenchus* species is important. Nematodes are primarily identified on light microscopic observations and measurements of morphological and morphometrical features mainly of males and females (Coomans *et al.*, 1978). Within the genus *Bursaphelenchus* the spicule is the most important morphological character for species differentiation, but the tail shape and number of lateral lines are also important (Braasch, 2001; Ryss *et al.*, 2005). I intercepted the two tail forms of *B. xylophilus*: *viz.*, the M-form with a mucron-like projection (from Canada) and the R-form, lacking this projection and having the rounded tail characteristic of the species (from Japan). This finding confirms that the M-form only occurs in North America, whereas the R-form is mainly distributed in East Asia, but was also found in Portugal (Braasch, 2004a). The R-form can clearly be

differentiated morphologically from the other species, although slightly mucronate forms may also be found in R-form populations (Braasch, 2004a). *Bursaphelenchus mucronatus* can only be differentiated from *B. xylophilus* by the form of the female terminus (Tomiczek *et al*., 2003). Although the morphological characters are similar, the M-form of *B. xylophilus* can also be differentiated from *B. mucronatus* (Braasch, 2001). I also detected the two genotypes of *B. mucronatus*. The East Asian and European genotype have almost identical morphology, although a slight difference in mucro shapes and lengths might be present (Braasch *et al.*, 1998). As a matter of fact, they can only be separated with ITS-RFLPs (Hoyer *et al*., 1998). *Bursaphelenchus doui* differs from *B. xylophilus* by its conoid female tail and from *B. mucronatus* by larger spicules and the straight area in the central part of the lamina (Braasch *et al*., 2004a). *Bursaphelenchus rainulfi* can be easily differentiated from the *B. xylophilus* group and *B. thailandae* by the number of lateral lines (2 *vs* 4). Equally, the spicules of *B. thailandae* and *B. rainulfi* can be easily used to distinguish these species from those of the *B. xylophilus* group.

The CDA of morphometrical data enabled discrimination of the five species by nine male and eight female characters. The morphometrical characters used for this analysis correspond partly with the main taxonomic-informative characters for the genus *Bursaphelenchus*: body length, stylet length, spicule length, a, c and c' values (Ryss *et al*., 2005). The spicule characters provided the most useful taxonomic information. CDA is an additional tool for species separation by morphometric data. The comparison of the morphometric data with published data revealed that the mean values of each character differed. These differences are possibly due to the difference of the number of observed specimens, the sample origin (from fungi culture or wood sampling), precision of measurement and the standard deviation of mean values.

In their extensive synopsis of the genus *Bursaphelenchus*, Ryss *et al*. (2005) recommended the spicule shape, the number of lateral lines, the number and arrangement of the male caudal papillae, presence of the vulval flap in the female and the shape of female tail as major features to be considered for separation of *Bursaphelenchus* species. In view of that I have added extra morphometric data to the identified species, especially with respect to the spicule morphometrics.

Molecular methods provide useful additional information for species separation and are essential to differentiate the M-forms of *B. xylophilus* originating from North America. Further, in the case of *B. mucronatus*, ITS-RFLP analysis enables the East Asian type to be differentiated from the European type (Hoyer *et al*., 1998). The ITS-RFLP patterns that I

obtained for the described five species conformed to those published earlier (Braasch & Burgermeister, 2002; Braasch *et al*., 2004a; Burgermeister *et al*., 2005b). The phylogenetic trees constructed from the D2D3 sequence data clearly supported the division of East Asian and European genotype groups of *B. mucronatus*. *Bursaphelenchus doui* grouped with *B. xylophilus* and *B. mucronatus*, all members of the xylophilus group, which was separated from *B. rainulfi* and *B. thailandae.* Earlier, Ye *et al.* (2007) proved that LSU provided the most phylogenetically informative dataset for *Bursaphelenchus* diversity studies.

Bursaphelenchus mucronatus is considered to be non-pathogenic (McNamara & Stoen, 1988); however, Kulinich *et al*. (1994) suspected the species to cause tree death in the Far East of Russia. Possible introduction of foreign provenances of *B. mucronatus* with wood packaging could pose a risk which is not controlled by the existing quarantine measures. Both *B. xylophilus* and *B. mucronatus* can be transmitted by the same type of vector beetles (*Monochamus* spp.) (Mamiya & Enda, 1972; 1979). Establishment of the nematode would be facilitated by the simultaneous presence of nematode and vector. The present study illustrates the danger of introducing these nematodes *via* the import of contaminated packaging material. The risk of introducing the pine wilt disease may even be enhanced if the beetle vector is simultaneously detected in the imported wood packaging materials. Thus, strict quarantine regulations are needed for preventing the importation of *B. xylophilus* and other non-indigenous *Bursaphelenchus* species to non-infected countries or regions. The combination of morphological and molecular observation for species description and diagnosis of species is strongly recommended for *Bursaphelenchus* species.

Chapter 5

Emergence of Monochamus alternatus*, the vector of* Bursaphelenchus xylophilus*, from* Pinus thunbergii *logs and the transmission of the nematodes through feeding wounds*[*]

[*] **Li, H.**, Shen, P., Fu, P., Lin, M. & Moens, M. (2007). Characteristics of the emergence of *Monochamus alternatus*, the vector of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), from *Pinus thunbergii* logs in Nanjing, China, and of the transmission of the nematodes through feeding wounds. *Nematology* 9, 807-816.

5.1 Introduction

Chapter 2 discussed the transmission of PWN among new host trees in East Asia primarily by the Japanese pine sawyer (JPS), *Monochamus alternatus*, through maturation feeding (Mamiya & Enda, 1972). The nematodes are transferred to feeding wounds as fourth-stage dispersal juveniles, which are found throughout the tracheal system of beetles, but are concentrated in the tracheae atrium from the first abdominal spiracles (Enda, 1994). After emergence of the beetles from host trees, the dispersal nematode juveniles leave the tracheal system, travel down the setae on the terminal abdominal sclerites and drop off (Kobayashi *et al.*, 1984). Exodus of juveniles from the beetle occurs mainly between 10 and 40 days after beetle emergence (Togashi & Sekizuka, 1982). Such juveniles can also invade the host trees *via* oviposition wounds made by adult beetles (Edwards & Linit, 1992; Arakawa & Togashi, 2002). The percentages of adult JPS carrying PWN, the number of PWN transmitted by JPS during maturation feeding, and the exit of PWN during the adult life stage of JPS, are additional essential factors in the occurrence of the disease (Shibata & Okuda, 1989). The transmission ability of nematodes by the beetles, which depends on the temporal transmission pattern and virulence of the nematodes, is one of the factors determining the epidemiology of disease (Jikumaru & Togashi, 2001).

It was found in Japan that the transmission pattern of *B. xylophilus* by *M. alternatus* can be described by an L-shaped or unimodal curve (Togashi, 1985; Shibata & Okuda, 1989). Transmission curves for *B. xylophilus* have not been identified in Chinese conditions, although several researchers (Zhao *et al.*, 1999; Chai *et al*., 2000; Jiang *et al*., 2002) made preliminary observations on a limited number of beetles.

To improve understanding of the dynamics of pine wilt disease initiation in China, I monitored the emergence of adult JPS from wilt-killed *P. thunbergii* logs and examined PWN transmission to pine twigs through maturation feeding during the 2004 and 2005 seasons in Nanjing, China. The objectives of the work here were to obtain information on: *i)* the dynamics of beetle emergence; *ii*) the frequency of beetles carrying nematodes; and *iii*) the transmission of nematodes through feeding.

5.2 Materials and Methods

5.2.1 Preparation of wood logs

In the periods October to December, 2003 and 2004, 15 and 30 *P. thunbergii* trees (25-30 years old) killed by PWN, were felled in the mountain region of Nanjing, Jiangsu

province, China. They were cut into 1.0-1.2 m long logs and transported to the campus of Nanjing Agricultural University. Logs were kept in a top-covered shed field cage (2 × 4 × 2 m) with side walls made of 0.3 cm metal mesh. They were watered once a week until the end of the observations, which took place daily from early April to the end of July 2004 and 2005.

5.2.2 Beetle emergence

During their period of emergence, beetles were caught and sexed daily and the ambient temperature and humidity were recorded. The number of dispersal juveniles carried by each beetle was estimated by a non-destructive method (Zhang *et al*., 1995). Beetles that emerged on the same day and had no nematodes visible on the hindwing or in the spiracular atrium such that the tracheal openings at the base of the atrium were clearly visible, *i.e*., beetle category 0 estimated to carry less than 100 dispersal juveniles in Zhang *et al.* (1995), were grouped. They were transferred collectively into cages (30 × 40 × 50 cm) constructed of 0.3 cm wire mesh, provided with a fresh twig of a 1-year-old *P. thunbergii* and kept at room temperature. The twigs were renewed daily. At renewal, the feeding wounds were sectioned from the twigs and nematodes were extracted from these sections in a modified Baermann funnel at room temperature during 24 h. Nematodes were extracted from successive twigs until the insect had died. Nematodes were eventually also extracted from dissected cadavers in a modified Baermann funnel at room temperature. They were collected and counted daily. The extraction was stopped when no nematodes emerged from the cadaver during two successive days.

5.2.3 Nematode transmission

Beetles with nematodes present on the hindwing or in the spiracular atrium such that the tracheal openings remained visible (= category 1 in Zhang *et al.* (1995) estimated to carry a moderate number (10,000-30,000) dispersal juveniles), and beetles with abundant nematodes in the spiracular atrium such that the tracheal openings were occluded (= category 2 in Zhang *et al.* (1995), estimated to carry a great number (20,000 or more) of dispersal juveniles), were transferred into individual cages (20 × 30 × 30 cm) constructed as above, provided with a fresh twig of a 1-year-old *P. thunbergii* and kept at room temperature. The twigs were renewed daily. Nematodes were extracted from the twigs and beetle cadavers as described above.

5.2.4 Statistics

One-way analysis of variance (ANOVA) and Tukey-Kramer's multiple comparison tests (S-PLUS 6.1 for Windows) were used to determine the significance of mean differences in the number of nematodes remaining in dead beetles of different ages.

5.3 Results

5.3.1 Emergence of nematode vector

The first *Monochamus* beetles emerged from the *P. thunbergii* logs from late April to early May of both years (Table 5.1).

Table 5.1 Dynamics of emergence of *Monochamus alternatus* adults from *Pinus thunbergii* logs in Nanjing, Jiangsu province, China in 2004 (15 trees) and 2005 (30 trees).

		2004	2005
Emergence period	Start	5 May	28 April
	End	25 June	26 June
	Duration (days)	51	60
	Number of beetles	438	927
Emergence peak period	Start	27 May	26 May
	End	11 June	11 June
	Duration (days)	16	17
	% of total	76.7	68.9
Sex ratio (male: female)		1.03	1

The full period of emergence of the beetles lasted between 51 and 60 days. During that time, the average temperature and humidity were 23.5ºC (19.0-31.0ºC) and 70.5% (55.0-90.0%) in 2004 and 24.9ºC (20.0-29.8ºC) and 72.4% (58.0-92.0%) in 2005. In 2004 and 2005, I collected 438 and 927 adults, respectively. Their sex ratio (1:1) was similar in both years.

5.3.2 Characteristics of PWN burden on JPS

Twenty-eight out of a total of 438 emerged beetles (6.4%), and 45 out of 927 beetles (4.9%) were classified as category 1 or 2 in 2004 and 2005, respectively. The other beetles were classified as category 0. From the cadavers of the beetles in this latter category, I extracted nematodes from 23 and 36% of the beetles in 2004 and 2005, respectively.

During the initial phase in 2004 only a few beetles emerged. However, the proportion of beetles that carried nematodes within the accumulated number of emerged beetles was high (Fig 5.1). At the start of the emergence peak, the proportion of beetles that carried nematodes within the accumulated number of emerged beetles decreased to less than 20% and remained at that level during the remainder of the observations. In 2005, only a small number of adults carried nematodes at the beginning of emergence. However, the percentage of beetles carrying nematodes increased steadily to about 30 and 40%, 8 and 14 days after the emergence of the first beetle, respectively (Fig 5.2).

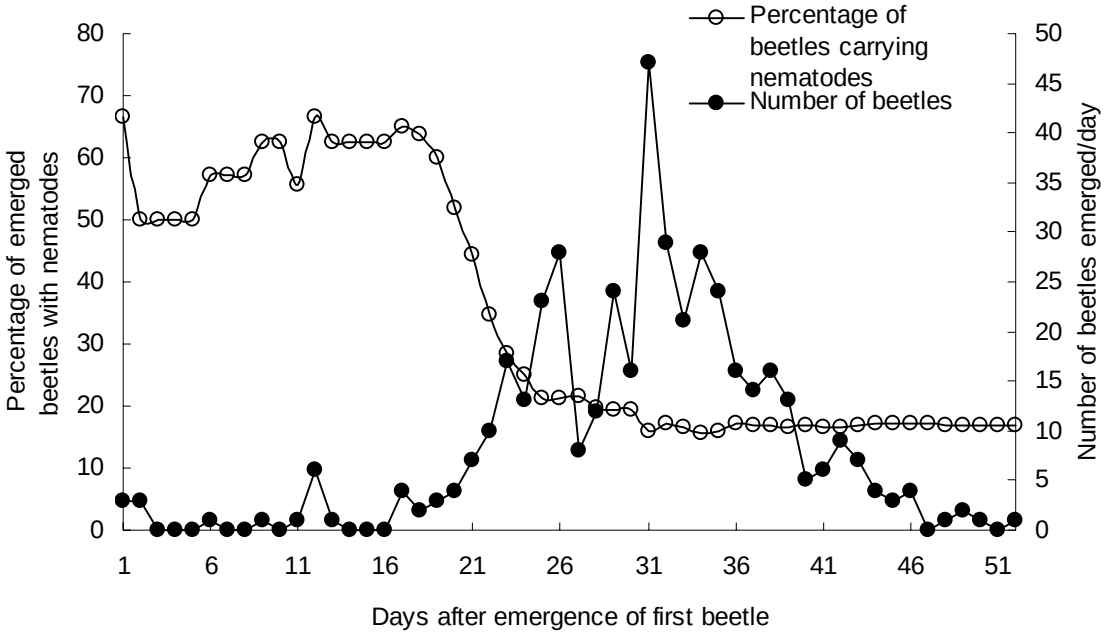

Fig 5.1 The dynamics of the percentage of *Monochamus alternatus* that carried *Bursaphelenchus xylophilus* during the emergence period in 2004.

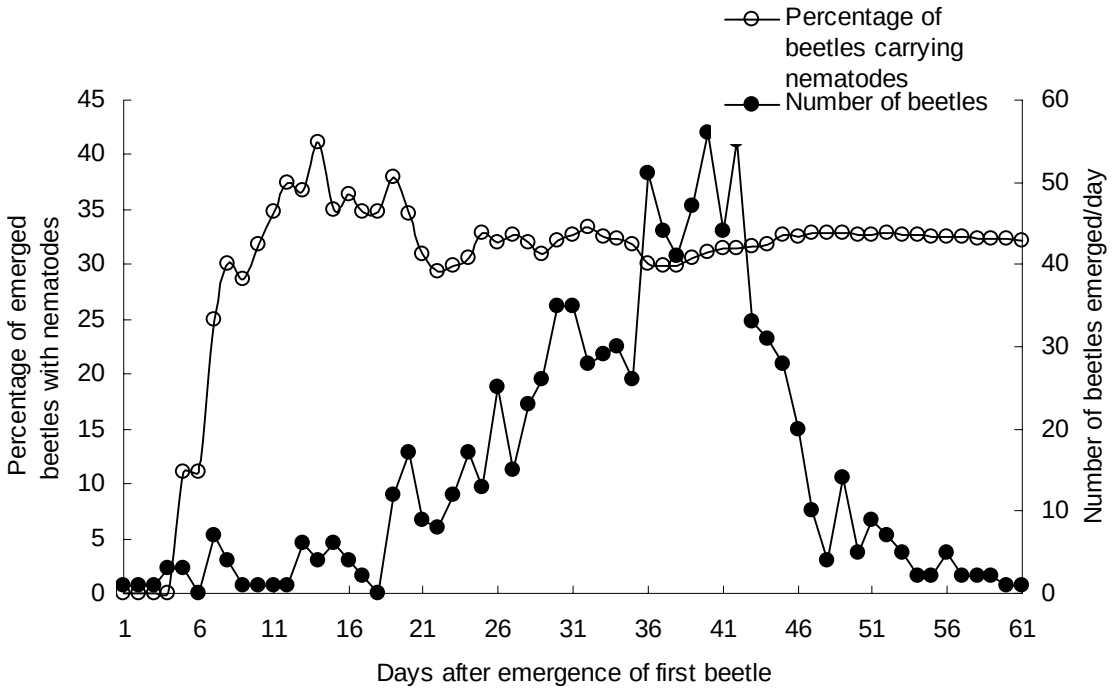

Fig 5.2 The dynamics of the percentage of *Monochamus alternatus* that carried *Bursaphelenchus xylophilus* during the emergence period in 2005.

If no nematodes were extracted from dead beetles during the first 24 h, further extraction during the following 48 and 72 h remained without success. However, if nematodes were extracted from the cadavers during the first 24 h, nematodes could still be extracted, sometimes up to 12 days. Only 35-40% of the nematodes present were extracted from the cadavers after 24 h; after 3 days this percentage increased to 70%. The greatest number of nematodes extracted from female and male beetles in one day was 1683 and 1200 in 2004, and 3240 and 4000 in 2005, respectively. No difference was found between the number of nematodes extracted from male and female beetle cadavers.

As the number of nematodes carried by a vector continues to decrease when the vector ages (Jikumaru & Togashi, 1995) and because the percentage of nematodes remaining within a dead adult tends to increase with the initial number of nematodes carried (Togashi, 1985), I used the number of nematodes that remained in a dead beetle to understand the relationship with beetle longevity. In 2004, 62% of the female and 79% of the male beetles without nematodes survived by feeding on twigs for no more than 10 days, whilst 32% of the females and 75% of the males with nematodes survived for less than 10 days (Table 5.2). There was no difference between the number of nematodes carried by female and male beetles ($F = 1.13$, $df = 1$, $P = 0.29$). Similarly, there was no significant difference between the numbers of nematodes remaining in cadavers of males and females of different ages (Male: $F = 2.085$, $df = 1$, $P = 0.163$; Female: $F = 0.316$, $df = 4$, $P = 0.865$).

Table 5.2 The relationship between the longevity of *Monochamus alternatus* (PWN) adults and their load of *Bursaphelenchus xylophilus* in 2004.

Longevity (days)	Females			Males		
	Number without PWN	Number with PWN	Mean number of PWN in cadavers	Number without PWN	Number with PWN	Mean number of PWN in cadavers
0-10	134	11	471.8 ± 796.2 a*	134	18	372.9 ± 603.4 a
11-20	7	6	432.1 ± 711.5 a	11	6	39.4 ± 25.4 a
21-30	23	11	685.2 ± 1047.0 a	7	0	
31-40	14	4	155 ± 166.5 a	2	0	
41-50	12	2	192.5 ± 187.4 a	3	0	
51-60	7	0		4	0	
61-70	7	0		5	0	
71-80	5	0		4	0	
81-90	8	0		0	0	

***** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

In 2005, 53% of the females and 54% of the males without nematodes survived no more than 10 days by feeding twigs, whilst 54% of the females and 67% of the males with nematodes survived less than 10 days (Table 5.3).

Table 5.3 The relationship between the longevity of *Monochamus alternatus* (PWN) adults and their load of *Bursaphelenchus xylophilus* in 2005.

Longevity (days)	Female			Male		
	Number without PWN	Number with PWN	Mean number of PWN	Number without PWN	Number with PWN	Mean number of PWN
0-10	122	74	420.0 ± 550.4 a*****	132	92	496.0 ± 1429.8 a
11-20	14	22	1056.0 ± 1327.5 a	21	14	774.0 ± 1395.2 a
21-30	21	10	1540.0 ± 1860.5 a	15	7	499.3 ± 474.6 a
31-40	7	8	2162.4 ± 1563.6 b	13	8	362.9 ± 451.6 a
41-50	5	4	1012.6 ± 273.6 b	8	4	179.0 ± 283.4 a
51-60	1	1	102 b	5	6	474.5 ± 372.0 a
61-70	10	1	820 b	13	2	464.0 ± 647.7 a
71-80	15	7	1754.7 ± 3054.8 b	17	2	619.0 ± 793.4 a
81-90	12	3	430.7 ± 450.5 a	10	1	232 a
91-100	16	5	247.0 ± 397.3 a	8	1	14 a
101-110	3	2	1914.5 ± 867.2 a	3	0	
111-120	2	0		1	0	
121-130	1	0		0	0	

***** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

The number of nematodes carried by female beetles was significantly different from that obtained from male beetles (F = 3.76, df = 1, P = 0.05). There was also a significant difference in the number of nematodes that remained in cadavers of females of different ages (F = 3.17, df = 10, P = 0.0012), but no difference in the numbers of nematodes that remained in males of different ages (F = 0.1242, df = 9, P = 0.999).

During both years of observations, the high death rate within the 10 days following the emergence of the beetles was similar, irrespective of whether they were loaded with nematodes or not. In 2004, the number of nematodes carried by both sexes of beetles had no influence on the longevity of the beetles. However, in 2005 the number of nematodes carried by females had a significant influence on their longevity.

5.3.3 Transmission of PWN into pine twigs through JPS feeding

The transmission of PWN to fresh pine twigs was examined in greater detail on a number of nematodes carrying beetles (15 in 2004 and 30 in 2005). Details on the transmission of nematodes by some of these individuals are listed in Table 5.4.

Table 5.4 Summary of *Bursaphelenchus xylophilus* transmission by a number of *Monochamus alternatus* feeding on twigs of *Pinus thunbergii.*

Code of beetles*	Detection of first nematodes (days after beetle emergence)	Total number of nematodes transmitted	Number of nematodes retained in dead beetle	Number of nematodes transmitted per day (max-min)	Last detection of nematodes (days after beetle emergence)	Number of days at which nematodes appeared in feeding wounds during whole feeding period
A1	10	193	0	69-1	74	11
A2	13	393	1301	116-1	77	23
A3	37	84	0	30-1	72	12
A4	17	994	33	239-1	79	35
A5	14	441	2520	158-1	61	16
B2	31	4	15	2-1	37	3
B4	26	7	1058	6-1	3	2
B5	17	354	220	343-1	23	4
B7	34	4	0	2-2	39	2
B8	10	1638	11	350-1	54	32
B9	32	61	0	12-1	49	16
B10	15	177	26	37-1	55	24
B11	44	5	5	3-1	23	3
B13	26	16	0	10-1	40	4
B15	12	3	0	3-3	3	1

* Code A: beetles emerged in 2005; B: beetles emerged in 2004.

The first nematodes were extracted from feeding wounds ten days after the beetles had emerged and started their maturation feeding (*e.g*., beetle A1); sometimes nematodes were detected for the first time only after more than one month (*e.g*., beetle B7). The period of nematode transmission could last for up to 79 days after beetle emergence (beetle A4). Nematodes could not be detected from feeding wounds every day. The maximum total number of nematodes transmitted to twigs by one beetle during the whole feeding period was 1638 (beetle B8); 2520 was the maximum number of nematodes extracted from a beetle after it died (beetle A5). The number of nematodes transmitted to twigs in 1 day varied between one and 350.

The dynamics of nematode transmission during the life span of beetles were expressed graphically and two types of the nematode-transmission curve were observed (Fig 5.3). In the unimodal pattern the nematode transmission peaked between 3-6 weeks after beetle emergence (beetles A4 and B10). A bimodal pattern that we observed (beetles B8 and A2), showed two transmission peaks: one between weeks 2-3, and another one between weeks 5-7 after beetle emergence.

5.4 Discussion

During their observations in 1991 and 1992 in the Nanjing region, Xu *et al.* (1994b) collected the first emerging *Monochamus* beetles during the first and second weeks of May. I detected the first *Monochamus* beetles during the period of late April to early May in both 2004 and 2005. The small difference in time with my data might be explained by an increase in temperature inducing an earlier emergence of the beetles. Xu *et al*. (1996) demonstrated that high temperatures stimulate the early emergence of *M. alternatus*, and Taniwaki *et al*. (2004) showed temperature to be the ruling factor for diurnal emergence.

In 2004 and 2005, I collected 438 and 927 adults, respectively. In the second year the beetles emerged from double the number of trees than in the first year, so these data suggest that a similar number of beetles emerged per tree in both years. The sex ratio of the emerged beetles (1:1) was also similar in both years. The similarity between both years is probably due to the similarity in both temperature and humidity. The peak emergence (68 or 76% of the total number of beetles) started 20 or 30 days after the emergence of the first beetle and continued from late May to early June, a period similar to that occurring in natural forest stands in Nanjing, Jiangsu (Xu *et al*., 1994b). Application of pesticides or traps baited with volatile lures in this period may significantly reduce the population of *M. alternatus* and decrease the mortality of pine trees caused by *B. xylophilus*.

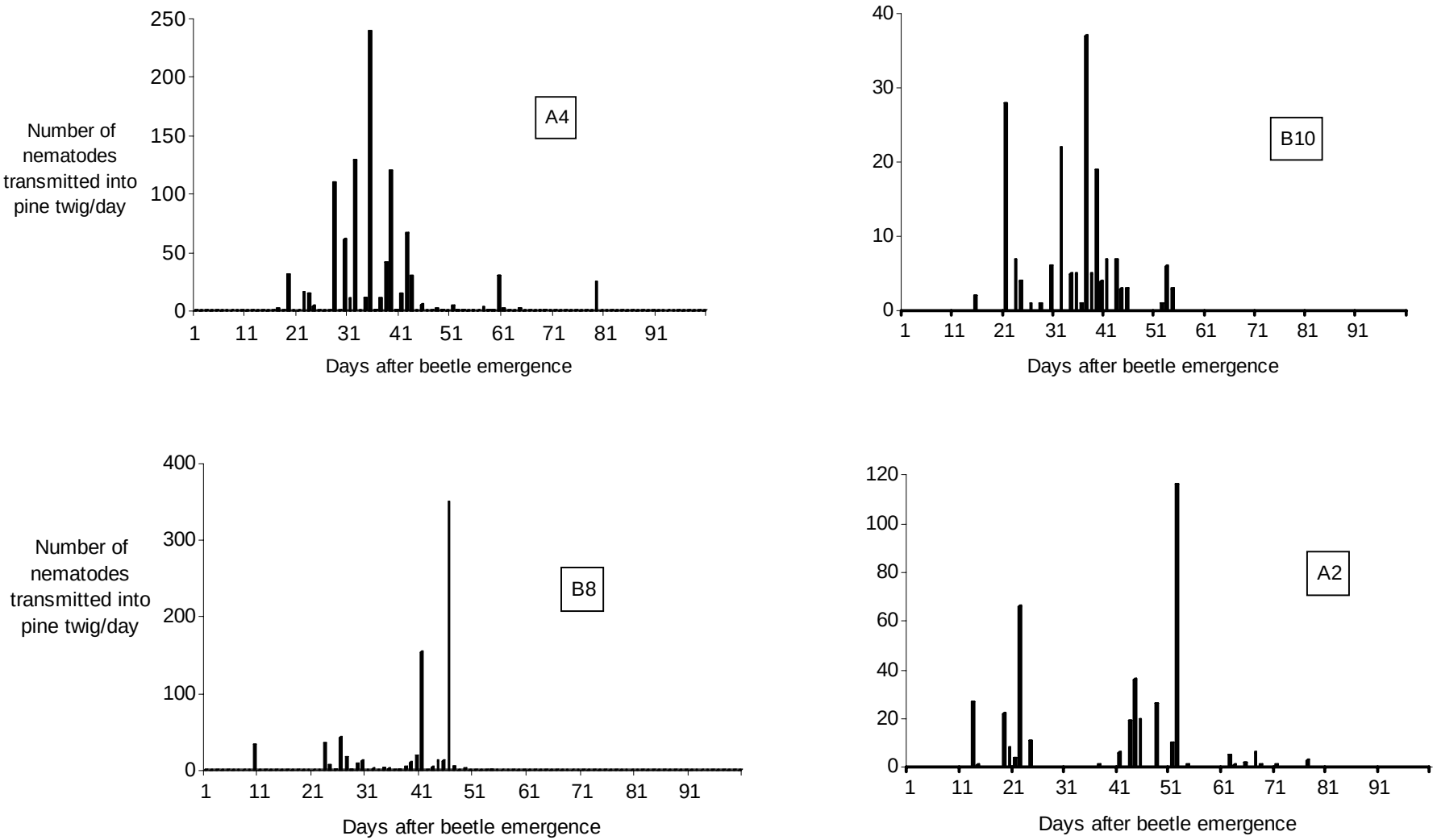

Fig 5.3 Four examples of the invasion of *Bursaphelenchus xylophilus* from individual adults of *Monochamus alternatus* to twigs of *Pinus thunbergii.* The nematode-transmission curve can be divided into two types, unimodal (beetles A4 and B10) and bimodal (beetles B8 and A2).

A good estimation of the nematode load just after emergence of the beetles is a prerequisite for the study of the interrelationship of the nematode, its vector and the plant host. Nematode number estimation would be redundant if precise nematode numbers could be inoculated reliably. However, Aikawa *et al*. (1997) found a large variation in the initial nematode load of beetles, even when they were loaded with the same artificial procedure. That makes the precision of an artificial inoculation with a preset number of nematodes doubtful. On the other hand, when using the Zhang *et al.* (1995) method to estimate the nematode load, I found that beetles were easily wounded by cutting off antennae or legs, or I injured spiracles and their atria. An approximate estimation of the initial nematode load is possible by a count of the fourth-stage dispersal juveniles on an artificial pupal chamber wall just after adult emergence (Aikawa & Togashi, 1997), but this is not practical for estimating nematode burden when large numbers of beetles emerge.

During my 2-year observations, the percentage of beetles that carried *B. xylophilus* was much lower than earlier reports of up to 75% of the population (Mamiya & Enda, 1972; Shibata & Okuda, 1989; Chai *et al*., 2000). One of the possible explanations for this difference might be the difference in humidity at which the logs were kept. Togashi (1989b) and Warren and Linit (1992) demonstrated that the moisture content of the wood is positively correlated with the population density of *B. xylophilus*. Fukushige (1990) confirmed this, and further showed that also the degree of blue stain fungi (on which the nematodes find an alternative food source) density is positively related to the water content of the host plant xylem.

During both years of observations, I observed a similar high death rate within the 10 days following the emergence of the beetles. In 2004, the nematodes load had no influence on the longevity of both sexes. In 2005, however, the nematodes influenced the longevity of the females. Togashi and Sekizuka (1982) came to the conclusion that the life-span of adult *M. alternatus* reared at room temperature was negatively correlated with the number of nematodes they carried. The large number of nematodes that could still be extracted from cadavers of long-living beetles suggests that *Monochamus* may transmit nematodes to pine trees through its whole life.

The graphical presentation of the dynamics of nematode transmission during the beetles' life yielded two types of nematode-transmission curve: a unimodal pattern and a bimodal pattern. The unimodal pattern was detected previously by Togashi (1985) when investigating the transmission of PWN to Japanese red pine (*P. densiflora*) in

Japan by measuring the number of nematodes transmitted into twigs at 5-day intervals. The author demonstrated that the transmission peak occurred during a period from days 20-35 after emergence of the beetle. The unimodal transmission was also found by Shibata and Okuda (1989) who observed the nematode transmission in the Nara and Mie Prefectures of Japan, and found peaks 2 and 5 weeks after beetle emergence, respectively. The bimodal pattern described here has never been observed before. It shows two transmission peaks: one between weeks 2-3, and another one between weeks 5-7 after beetle emergence. My daily examinations of transmission might have yielded more precise data, thus allowing for the identification of a second transmission curve. The data clearly reveal the existence of a transmission period longer than previously accepted.

Togashi (1985) showed that the peak of the *B. xylophilus* transmission curve was greater when the initial nematode load increased. The author further showed that the percentage of nematodes remaining within a dead vector tended to rise when the initial nematode load increased. Shibata and Okuda (1989) confirmed that the main factors affecting the number of nematodes transmitted to pine twigs are the number of nematodes carried by adult beetles and the longevity of the beetle. Similarly, Jikumaru and Togashi (2001) proved that the initial nematode load, the nematode departure efficiency and the nematode transmission efficiency had significantly positive influences on the number of nematodes transmitted into pine twigs. Yamane *et al.* (2004) further demonstrated that the numbers of nematodes that migrated from individual JPS in wet twigs were greater than in dry ones, and temperature reduction activated nematodes movement. High transmission efficiency is necessary for the pathogen to persist at low host density (Jikumaru & Togashi, 1995). Overall, the epidemiology of pine wilt disease through beetle maturation feeding and oviposition is directly related to the initial number of nematodes being carried after beetle emergence and is also related to the various physical, chemical and environmental factors, particularly temperature and moisture. However, a precise and practical method for estimating the initial number of nematodes remains to be developed.

Two years of observations of the biology of *M. alternatus* carried out in Jiangsu Province revealed that in this area beetle emergence starts late April, peaks from late May to early June and stops by the end of this month. The frequency of beetles carrying PWN was between 20 and 40% of the total number of emerged beetles and large numbers of nematodes were detected in beetles after a long life span. These data

suggest that if chemical control were to be an option for controlling the vector beetle, it should start earlier than previously thought and be continued for a long period. This makes the chemical control strategy even less attractive than it is already.

Artificial inoculation tests with PWN often yielded highly variable results in different reports (McNamara, 2004). In view of my results demonstrating that the number of nematodes transmitted to twigs in one day may vary between one and 350 individuals, one can conclude that replacing the artificial inoculation by a natural inoculation is not an immediate option. Inoculations of PWN should be done in precise quantities similar to the number of beetles transmitted in nature.

Chapter 6

*Pathogenicity tests of pine wood nematode (*Bursaphelenchus xylophilus*) on* Pinus thunbergii *and other* Pinus *spp.*

6.1 Introduction

The most effective method for PWN control is planting resistant *Pinus* or other coniferous species and their varieties. Resistance screening systems depend on the aggressiveness of the nematode populations as well as on their pathogenicity to plants (Ikeda, 1984). The pathogenicity of *B. xylophilus* to *Pinus* species has been intensively investigated under both field and laboratory conditions but has often yielded very variable results (Mamiya, 1983; Bedker *et al.*, 1987; Linit & Tamura, 1987; Bedker & Blanchette, 1988; Panesar & Sutherland, 1989; Schauer-Blume, 1990; Riga *et al*., 1991; Braasch, 1997).

McNamara (2004) criticized the techniques used in the reported pathogenicity screening tests. Differences in nematode populations, nematode densities for inoculation, life stages of the inoculated nematodes, ages of the inoculated plants, environmental conditions and inoculation techniques, could affect the results of pathogenicity tests and these factors may led to great variations among different reports of pathogenicity. The author confirmed that the testing methods had provided very variable results whilst the experiments did not give any confidence in the relevance of the results in relation to field conditions. The author further stated that pathogenicity studies with *Bursaphelenchus* species are notoriously difficult to perform accurately due to the difficulties in simulating natural inoculation with beetle vectors. The vector may provide cues to the nematode dauer stage which prepare it for infection and, since nematodes harvested from PDA plates will not have had exposure to these cues, they may not be as suitably prepared for infection of plants. McNamara concluded that there was an urgent need for in-depth research into the criteria which determine the response of *Pinus* plants after inoculation with nematodes in relation to various factors, such as age of host plant, method of inoculation (including use of vector inoculation), state of sterility of the nematode suspension, life stage of the nematodes, environmental conditions, *etc*. in order to find a means to relate such experiments to natural conditions of infection and disease expression.

The aim of the research on which I report in this chapter was to evaluate the effect of various factors on the pathogenicity of *B. xylophilus* to *P. thunbergii,* and to evaluate the pathogenicity of different *B. xylophilus* populations to different *Pinus* spp. Inoculation tests were designed and included the simulation of natural nematode infection through beetle vectors and the artificial inoculation of dispersal juveniles from beetles as well as nematodes cultured in different ways on *Botrytis cinerea*.

6.2 Materials and methods

6.2.1 Pathogenicity of *Bursaphelenchus xylophilus* on *Pinus thunbergii* by artificial and natural methods

6.2.1.1 Plants

During each year of experiments healthy PWN free *P. thunbergii* seedlings 3-4 years old were transplanted in a fully randomised block design into the experimental field in Jiangning, Nanjing three months prior to PWN inoculation. Healthy PWN free *P. thunbergii* trees of 7-9 years old were planted in the same experimental design on the slope of the hill region close to the experimental field. The experiments were run for four months. No pine wilt disease had occurred in recent years in the area where the plants were grown.

6.2.1.2 Nematodes and artificial inoculation

Previously wilting dead pine trees were felled in the mountain regions of Lianyungang, Jiangsu province, China during the periods October-December of 2003-2005. They were cut into 1-1.2m long logs and kept in metal mesh cages at the campus of Nanjing Agricultural University (see chapter 5).

Adults of *M. alternatus* emerging from the logs were collected in 2004 and 2005. Some dispersal juveniles were extracted directly from these beetles by modified Baermann funnels and were used as inoculum (BDJ, dispersal juveniles from beetle) in different tests. Other juveniles were collected from beetles or logs and cultured and maintained on *B. cinerea* (see chapter 3) and used as inoculum.

Some dispersal juveniles that were collected from beetles (B) were cultured for a single generation on *B. cinerea*. They were either surface sterilized (Zhao *et al*., 2003) before being used as inoculum (BFS), directly used as inoculum (BFNS), or heat killed at 60ºC for 10 min before they were used as inoculum (BHK). Some of the dispersal juveniles were kept on *B. cinerea* for multiple generations and eventually used as inoculum without surface sterilization (BFNSM).

Dispersal juveniles collected from dead wood logs (W) were cultured on *B. cinerea.* Some were cultured for only one generation. These were used as inoculum either after sterilisation (WFS), or without sterilisation (WFNS), or after being heat-killed (WHK). Others were cultured for multiple generations before they were inoculated without sterilisation (WFNSM). SDW without nematodes was used in the control (CK).

In 2004 and 2005 BDJ were inoculated at different densities. 200, 400, 600, 800 or 1000 nematodes per plant in 2004; 5, 50, 100, 150 or 200 nematodes per plant in 2005. In 2004 1000 nematodes from other sources were used in other inoculations. In the experiments of 2005 to 2007, a density of 200 nematodes per plant was used in all tests. In all experiments, nematode suspensions were adjusted to appropriate volumes depending on the number of nematodes to be inoculated to each plant. The number of nematodes in the initial suspension was estimated by taking five aliquots of 0.1 ml, counting the nematodes contained in each of them and calculating the mean.

Inoculations were carried out late June of 2004 to 2007. To optimise the success of the inoculations, nematodes were inoculated in the late afternoon, thus avoiding the high temperatures that occur during summer in Nanjing. Nematodes were inoculated to the stem of 3-year old seedlings or to the base of the youngest branch of the 7-9-year old trees. The needles around the inoculation point were removed and a slit of about 1cm in length was cut in the bark using a scalpel. A small piece of cotton wool was inserted slightly under the bark. The nematode suspension was dropped slowly on the cotton wool with a pipette. Finally, the inoculation site was sealed off with a plastic strip to prevent desiccation (Braasch, 1996). Plants in the control treatment received a similar inoculation procedure in which SDW replaced the nematodes.

6.2.1.3 Natural inoculation by beetles

In 2004 beetles that had emerged from the caged logs and were classified in category 1 or 2 of Zhang *et al.* (1995) (see chapter 5) were used for BDJ inoculation. Fifteen beetles were selected and individually reared for two weeks on excised 1-year-old fresh twigs of *P. thunbergii* in cages made of steel mesh (2 × 2 mm apertures). The beetles were then divided into three groups for feeding on plants for 1, 2 or 3 days. Each beetle was individually caged in bags made of plastic mesh (2 × 2 mm apertures) on the stem of 3-year-old seedlings *of P. thunbergii* or branch of 7-9-year-old *P. thunbergii* trees in the late afternoon of 20 June of 2004. The beetles were removed from the plants after the preset feeding times and the feeding wounds were checked. Each beetle was dissected immediately and nematodes left inside the beetle cadaver were extracted for 24 hr in a modified Baermann funnel; the total number of nematodes was calculated.

In 2005, adult beetles that emerged during the four days peak period were used for inoculation. Beetles that had emerged during the same day were reared together on pine twigs

in cages made of steel mesh (2 × 2 mm apertures). After two weeks, 66 beetles were individually caged with plastic mesh bag on the stem of 3-4-year-old young seedlings of *P. thunbergii* in the late afternoon of 28 June. Twenty-four hours later the feeding wounds were checked, the beetles removed and nematodes extracted as described above.

6.2.1.4 Symptom observation

The plants were watered when necessary. Wilt symptoms were checked at 2-week intervals from July to September. The symptoms were categorised according to Malek and Appleby (1984). Five stages were classified as follows: 1: only needles around the inoculated place are yellowish, needles in the other part are green; 2: needles in the upper and lower part of the inoculation spot are brown yellowish, and needles in the top of the tree are greyish green; 3: needles in the upper and lower part of the inoculation spot are brown yellowish, and needles in the top are yellowish green; 4: all needles of the plant are yellowish brown; 5: all needles are brown. Healthy plants with green needles were classified as stage 0. Disease incidence was calculated using the formula (Fang, 1998):

$$\text{Disease incidence} = \frac{\sum (\text{Number of diseased plants} \times \text{Symptom stage})}{\text{Total number of plants} \times \text{Highest symptom stage}}$$

The disease incidence was calculated using symptoms observed six weeks after inoculation. Nematodes were extracted from individual plants as soon as they had died. At the end of the experiments, the remaining living plants were cut and checked for nematode presence even in the absence of wilt symptoms.

To re-isolate nematodes from the plants, stems and/or branches were cut. In the experiments of 2004, soil and the needles were removed from the dead plants; short shoots or thin branches were removed as well. Four parts (root, first branch division, second branch division, and top) or three parts (root, medium trunk and top with inoculation place) of young seedlings and 7-9 year old plants, respectively, were separated, weighed, chopped to small pieces (maximum length 1cm) and nematodes extracted using the Baermann funnel technique (see chapter 3). The fresh plant tissues weighed around 3-5 grams from young seedlings and 7-10 grams from 7-9 year old plants. The average numbers of nematodes per gram fresh tissue were calculated after 24 hr. In view of the results obtained in the 2004 experiments, the nematode re-isolation was modified in the tests from 2005 - 2007. Dead plants were cut off at

2 cm above soil level, and only the medium stem part was taken and used for 24 hr nematode extraction. The number of re-isolated nematodes was calculated per gram of fresh wood.

6.2.2 Pathogenicity of *Bursaphelenchus xylophilus* populations on *Pinus thunbergii* and three other *Pinus* species

During the first experiments, natural inoculation through beetles did not yield reliable results. The number of nematodes transmitted to plant was impossible to determine and the results were variable. Therefore, artificial inoculation methods were used for comparing the pathogenicity of populations of *B. xylophilus*.

6.2.2.1 Plants

Healthy, PWN-free, 3-4-year-old seedlings of *P. thunbergii*, *P. massoniana*, *P. taeda* and *P. elliotti* were transplanted into the experimental farm of Dongshanqiao, Nanjing three months before nematode inoculation in 2005, 2006 and 2007. Healthy, 7-9 year old PWN free, *P. thunbergii* trees were planted on the slope of the hill close to the experimental field in 2006. In the years prior to the experiments, no pine wilt disease was registered in the area where the experiments took place or in the area where the seedlings had been grown.

6.2.2.2 Nematodes populations

Five isolates of *B. xylophilus* originating from different regions in China and abroad (Table 6.1) were selected for pathogenicity tests on the *Pinus* species.

Table 6.1 Origins of *Bursaphelenchus xylophilus* populations used in the pathogenicity tests in the field.

Population	Origin	Host from which the nematode was isolated
BxLYG	Liangyungang, Jiangsu, China	*P. thunbergii*
BxSD	Changdao, Shandong, China	*P. thunbergii*
BxCAN	Canada	Unknown
BxJAP	Japan	*P. thunbergii*
BxPOT	Portugal	*P. sylvestris*

Prior to their use as inoculum, the nematodes were multiplied on *B. cinerea* and nematodes were not surface sterilised prior to inoculation. The methods for nematode extraction and calculation of nematode densities were as described in 6.2.1.2. Two hundred nematodes were inoculated into each plant. Plants in the control treatment received a similar inoculation procedure in which SDW replaced the nematodes.

6.2.2.3 Inoculation and symptom observation

The plants were inoculated by bark-cutting (see 6.2.1.2) followed by the application of 200 *B. cinerea*-cultured nematodes without previous surface sterilisation. Symptom development was observed at two-week intervals and the disease incidences were calculated for each observation as described in 6.2.1.4. The mortality of plants was calculated for all observations. The nematodes were re-isolated as described in 6.2.1.4.

To compare the results obtained in the inoculation tests in relation to the success of the nematode populations in the inoculated *Pinus* species, a relative host suitability index (RHS) was calculated for evaluating the nematode populations (Braasch, 1997). The RHS formula is RHS= death rate of plants after inoculation × average number of nematodes extracted from per gram plant tissue /1000.

6.2.3 Statistical analysis

One-way analysis of variance (ANOVA) and Tukey-Kramer's multiple comparison tests (S-PLUS 6.1 for Windows) were used to determine the significance of mean differences in survival of plants and the number of nematodes recovered from dead plants between different inoculation techniques and densities.

6.3 Results

6.3.1. Effect of *Bursaphelenchus xylophilus* on *Pinus thunbergii*

During the four years of experiments, wilting usually appeared on both 3-4-year-old seedlings and 7-9–year-old plants two weeks after PWN inoculation in the compatible host - nematode interactions. Release of resin was clearly reduced on susceptible plants and some needles became greyish-green. After four weeks, some needles of inoculated susceptible trees became yellowish-brown. Some plants died six weeks after inoculation. None of the non-inoculated control plants showed wilt symptoms or were killed during the observation period.

During the 2004 experiment, I did not observe significant differences in the number of nematodes isolated from the different plant parts from either 3-4-year old seedlings or 7-9-year old plants. Therefore, I decided for the experiments in 2005, 2006, and 2007 to estimate the number of nematodes from dead trees from the middle part of the 3-4-year old seedlings or 7-9-year old plants after a 24 hr Baermann funnel extraction.

6.3.1.1 Artificial inoculation

6.3.1.1.1 Three-four-year old plants

DISPERSAL JUVENILES AT DIFFERENT DENSITIES

In the 2004 experiment, the different nematode densities (200, 400, 600, 800 or 1000 BDJ per plant) developed different dynamics in symptoms and mortality of the 3-4-year old seedlings (Table 6.2). The survival time of the plant after inoculation was significantly influenced by the inoculation densities ($F = 6.47$, $df = 4$, $P < 0.0001$); equally, the number of nematodes recovered from dead tissues differed between the nematode densities ($F = 3.13$, $df = 4$, $P = 0.016$).

Table 6.2 Effect of artificial inoculation with nematode densities (number of nematodes per seedling) of *Bursaphelenchus xylophilus* on 3-4-year seedlings of *Pinus thunbergii* in 2004**.**

Dose	Plants tested	Plants killed	Disease incidence**	Mortality (%)	Plant survival time (days)	Nematodes per gram fresh wood tissue
200	14	12	0.50	85.7	54.8 (40-75) a***	379.2 (0-4857) a
400	8	7	0.75	87.5	59.4 (48-63) a	316.5 (8.5-1132.9) a
600	8	7	0.53	87.5	51.9 (42-63) a	536.2 (5.4-2280.0) a
800	8	6	0.58	75.0	54.7 (45-63) a	214.6 (5.6-1326.0) a
1000	8	7	0.47	87.5	62.4 (59-75) a	959.1 (1.5-6082.7) a
CK*	8	0	0	0		

*****CK: untreated control
****** Disease incidence calculated 42 days after plants inoculated nematodes at 20th June, 2004.
******* With each column, means followed by the same letter are not significantly different from each other according to Tukey Test ($P<0.05$).

In the 2005, no seedling had died before the end of the experiment at any of the inoculation densities. The effect of the nematodes was restricted to a few plants showing yellowish needles.

FUNGAL-CULTURED NEMATODES ISOLATED FROM *MONOCHAMUS* BEETLES AND TREATED WITH DIFFERENT METHODS BEFORE INOCULATION

In 2004, *Pinus*-seedlings were inoculated with 1000 nematodes that had received different treatments. Significant differences between treatments were recorded in plant survival time ($F = 38.3$, $df = 2$, $P < 0.0001$) and in number of nematodes recovered from dead wood tissue at the end of the experiment ($F = 5.75$, $df = 2$, $P = 0.004$). Seedlings inoculated with fungus-cultured nematodes without surface sterilisation (BFNS) expressed wilt

symptoms much faster and showed a higher mortality when compared to treatments with nematodes that were surface sterilised (BFS) or the inoculation with dispersal juveniles (BDJ) (Table 6.3). Six weeks after inoculation of the nematodes, the disease incidence of BFNS nematodes was 0.93 and all plants had died 60 days after inoculation.

Table 6.3 Effect of inoculation (1000 nematodes/plant) of *Bursaphelenchus xylophilus* on 3-4-year old seedlings of *Pinus thunbergii* in 2004. Nematodes were isolated from *Monochamus* adults, cultured on *Botrytis cinerea* or not and exposed to different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Disease Incidence**	Mortality (%)	Plant survival time (days)	Nematodes per gram of fresh wood tissue
BDJ*	8	7	0.47	87.5	62.4 (59-75) a***	959.1 (1.5-6082.7) a
BFS	14	10	0.48	71.4	61.0 (47-83) a	374.8 (15.1-1848.1) b
BFNS	14	14	0.93	100.0	50.0 (44-60) b	302.0 (0-1343.4) b
CK	15	0	0	0		

* BDJ: dispersal juveniles isolated straight from beetles; BFS: nematodes cultured on *Botrytis cinerea* and surface sterilised before inoculation; BFNS: nematodes cultured on *Botrytis cinerea* but not surface sterilised; CK: untreated control.
** Disease incidence calculated 42 days after plants inoculated nematodes at 20th June, 2004.
*** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

In view of the results obtained during the previous year, the number of nematodes inoculated onto seedlings in 2005 was decreased to 200 nematodes per plant. Significant differences between treatments were found in plant survival time (F = 49.6, df = 2, P < 0.0001). With respect to the number of nematodes recovered from dead tissues no significant difference between BFNS and BFS was recorded (F =0.98, df = 1, P = 0.34). The non-sterilised nematodes (BFNS) caused faster wilting than the sterilised nematodes (BFS) (Table 6.4). The disease incidences were 0.82 and 0.38 for BFNS and BFS, respectively. All BFNS-treated plants died within 58 days after inoculation; of the BFS-treated plants only three out of ten seedlings had died 58 days after inoculation. No plant died when inoculated with nematodes that were killed by heating (BHK), only some of them showed minor wilt symptoms (yellowish needles).

Table 6.4 Effect of inoculation (200 nematodes/plant) of *Bursaphelenchus xylophilus* on 3-4-year old seedlings of *Pinus thunbergii* in 2005. Nematodes were previously isolated from *Monochamus* adults, cultured on *Botrytis cinerea* and exposed to different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Disease Incidence **	Mortality (%)	Plant survival time (days)	Nematodes per gram of fresh wood tissue
BFS*	10	3	0.38	30.0	58 (58-58) a***	1028.3 (442-1613.7) a
BFNS	13	13	0.82	100.0	43.8 (35-58) a	2965.6 (38.9-7308.1) a
BHK	15	0	0.19	0	0 b	
CK	15	0	0	0		

* BFS: nematodes cultured on *Botrytis cinerea* and surface sterilised before inoculation; BFNS: nematodes cultured on *Botrytis cinerea* but not surface sterilised; BHK: heat killed nematodes cultured on *Botrytis cinerea*; CK: untreated control.
** Disease incidence calculated 42 days after plants inoculated nematodes at 17th June, 2005.
*** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

In 2006, there were no significant differences between plant survival time (F = 2.094, df = 2, P = 0.1393) and numbers of nematodes recovered from dead tissues (F = 1.699, df = 2, P = 0.1984) as influenced by the inoculations with the different nematode treatments (Table 6.5). However, BFNS-inoculated plants showed faster wilting compared to those inoculated with nematodes collected from wood logs and cultured for a single (WFNS) or multiple cycles (WFNSM).

Table 6.5 Effect of inoculation (200 nematodes/plant) of *Bursaphelenchus xylophilus* on 3-4-year old seedlings of *Pinus thunbergii* in 2006. Nematodes were collected from wood logs or beetles, cultured on *Botrytis cinerea* during a single or multiple cycles, and exposed to different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Disease Incidence**	Mortality (%)	Plant survival time (days)	Nematodes per gram of fresh tissue
WFNS*	15	12	0.63	80.0	56.2 (42-88) a***	1359.6 (3.6-5555.2) a
WFNSM	15	14	0.56	93.3.0	58.2 (42-88) a	1150.4 (8.1-7520.4) a
BFNS	14	14	0.8	100.0	48.3 (42-69) a	241.1 (2.9-1470.4) a
CK	15	0	0	0		

* WFNS: nematodes isolated from wood logs, cultured on *B. cinerea* during one cycle and non-surface sterilised before inoculation; WFNSM: nematodes isolated from wood logs, cultured on *B. cinerea* during several cycles and non-surface sterilised before inoculation; BFNS: nematodes isolated from *Monochamus* adults, cultured on *Botrytis cinerea*, but non-surface sterilised before inoculation; CK: untreated control.
** Disease incidence calculated 42 days after plants inoculated nematodes at 20th June, 2006.
*** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

In the 2007 experiment, the dynamics of the disease was monitored by the disease incidence (Fig. 6.1). The experiment included inoculations with nematodes isolated from wood logs and cultured for a single cycle on *B. cinerea* followed by surface sterilisation (WFS) or by heat-killing (WHK) prior to inoculation. Nematodes collected from beetles and maintained through multiple cultures on *B. cinerea* (BFNSM), were added to WFNS- and WFNSM-treatments as in 2006.

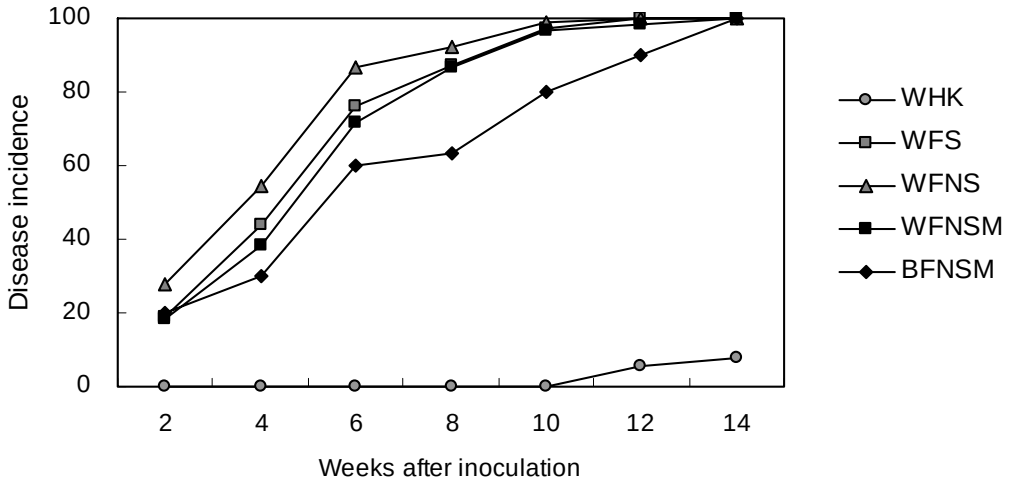

Fig 6.1 Symptom development (expressed as disease incidence) caused by inoculations (200 nematodes/plant) with differently treated *Bursaphelenchus xylophilus* on 3-4-year seedlings of *Pinus thunbergii*. WFS: nematodes isolated from wood logs and cultured for a single cycle on *Botrytis cinerea* followed by surface sterilisation; WHK: nematodes isolated from wood logs and cultured during a single cycle on *Botrytis cinerea* followed by heat-killing; BFNSM: nematodes collected from beetles and cultured for several cycles on *B. cinerea*; WFNS: nematodes isolated from wood logs, cultured on *B. cinerea* for one cycle and non-surface sterilised; WFNSM: nematodes isolated from wood logs, cultured on *B. cinerea* for several cycles and non-surface sterilised.

Six weeks after inoculation, WFNS-inoculated plants showed faster symptom development compared to WFS, WFNSM and BFNSM (Fig 6.1; Table 6.6). There was a significant difference between treatments with respect to plant survival ($F = 7.08$, $df = 4$, $P < 0.0001$). There was no significant difference between inoculations with WFS, WFNS, WFNSM and BFNSM in number of nematodes isolated from dead tissues ($F = 1.475$, $df = 3$, $P = 0.2318$). No BHK-inoculated plant died within the period of the observations, only few plants showed a few yellowish needles after 3 months.

Table 6.6 Effect of inoculation (200 nematodes/plant) of *Bursaphelenchus xylophilus* on 3-4-year old seedlings of *Pinus thunbergii* in 2007. Nematodes were collected from wood logs or beetles, cultured on *Botrytis cinerea* during a single or multiple cycles, and exposed to different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Disease incidence**	Mortality (%)	Plant survival time (days)	Nematodes per gram of fresh tissue
WFS*	16	16	76.3	100.0	84.8 (49-114) a***	1186.5 (46.2-3687.9) a
WFNS	30	28	86.7	93.3	74.7 (49-114) a	641.3 (6.1-2974.1) a
WFNSM	12	11	71.7	91.7	80.7 (68-114) a	744.1 (22.9-1857.5) a
BFNSM	6	5	60	83.3	113.1 (84-140) b	932.2 (143.8-1705.58) a
WHK	15	0	0	0	0 c	
CK	15	0	0	0		

* WFS: nematodes isolated from wood logs, cultured on *B. cinerea* during one cycle and surface sterilised; WFNS: nematodes isolated from wood logs, cultured on *B. cinerea* during one cycle and non-surface sterilised before inoculation; WFNSM: nematodes isolated from wood logs, cultured on *B. cinerea* during several cycles and non-surface sterilised before inoculation; BFNSM: nematodes isolated from *Monochamus* adults, cultured on *Botrytis cinerea* during several cycles, but non-surface sterilised before inoculation; WHK: nematodes isolated from wood logs, cultured on *B. cinerea* during one cycle and heat killed prior inoculation; CK: untreated control.
** Disease incidence calculated 42 days after plants inoculated nematodes at 19th June, 2007.
*** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

6.3.1.1.2 Seven-nine-year old plants

In 2004, inoculations with 1000 nematodes/plant included: (i) BDJ-, (ii) BFS- and (iii) BFNS-treated nematodes. The symptom development was faster on BDJ-inoculated plants than on BFS- and BFNS-plants (Table 6.7); significant differences in plant survival were observed between nematode treatments (F = 9.70, df = 2, P = 0.00014). There was no significant difference in numbers of nematodes recovered from dead plants between the different treatments (F = 1.93, df = 2, P = 0.15).

Table 6.7 Effect of inoculation (1000 nematodes/plant) of *Bursaphelenchus xylophilus* on 7-9-year old plants of *Pinus thunbergii* in 2004. Nematodes were isolated from *Monochamus* adults, cultured on *Botrytis cinerea* or not, and exposed to different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Disease Incidence**	Mortality (%)	Plant survival time (days)	Nematodes per gram fresh tissue
BDJ*	13	11	0.71	84.6	53.4 (49-66) a***	1089.7 (47.3-3035.8) a
BFS	13	12	0.65	92.3	62.2 (49-83) b	1876.9 (3.3-8978.4) a
BFNS	14	13	0.69	92.9	61.5 (49-83) b	1494.8(95.8-9417.8) a
CK	15	0	0	0		

* BDJ: nematodes in dispersal stage collected from adult *Monochamus*; BFS: nematodes cultured on *Botrytis cinerea* and surface sterilised before inoculation; BFNS: nematodes cultured on *Botrytis cinerea* but not surface sterilised; CK: untreated control.
** Disease incidence calculated 42 days after plants inoculated nematodes at 20th June, 2004.
*** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

In 2005, the inoculum density was reduced to 200 nematodes in view of the results obtained the year before. The symptom development and mortality of BFNS-plants were similar to that of BFS-plants. BHK-plants did not show wilt and did not die. Significant differences between nematode treatments were recorded on plant survival ($F = 9.70$, $df = 2$, $P = 0.00014$) and numbers of nematodes recovered from dead plants ($F = 13.4$, $df = 2$, $P < 0.0001$) (Table 6.8).

Table 6.8 Effect of inoculation (200 nematodes/plant) of *Bursaphelenchus xylophilus* on 7-9-year old plants of *Pinus thunbergii* in 2005. Nematodes were previously isolated from *Monochamus* adults, cultured on *Botrytis cinerea* and exposed to different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Disease Incidence**	Mortality (%)	Plant survival time (days)	Nematodes per gram fresh tissue
BFS*	15	5	0.27	33.3	62 (56-66) a***	4588.8 (51.3-9773.9) a
BFNS	15	4	0.31	26.7	58 (55-62) a	3049.4 (42.5-7966.8) a
BHK	15	0	0	0	0 b	0 b
CK	15	0	0	0		

* BFS: nematodes cultured on *Botrytis cinerea* and surface sterilised before inoculation; BFNS: nematodes cultured on *Botrytis cinerea* but not surface sterilised; BHK: heat killed nematodes cultured on *Botrytis cinerea*; CK: untreated control.
** Disease incidence calculated 42 days after plants inoculated nematodes at 17th June, 2005.
*** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test ($P<0.05$).

In 2006, inoculations using 200 nematodes/plant included: (i) BFNS-, (ii) WFNS- and (iii) WFNSM-treated nematodes. The symptom development was faster on BFNS-inoculated plants than on WFNS- and WFNSM-inoculated plants (Table 6.9); higher mortality was also observed on these plants. However, there were no significant differences in survival time ($F = 1.0479$, $df = 2$, $P = 0.365$) nor in the number of nematodes recovered from dead plants ($F = 0.960$, $df = 2$, $P = 0.396$) between the treatments.

Table 6.9 Effect of inoculation (200 nematodes/plant) of *Bursaphelenchus xylophilus* on 7-9-year old plants of *Pinus thunbergii* in 2006. Nematodes were collected from wood logs or beetles, cultured on *Botrytis cinerea* during a single or multiple cycles, and exposed to different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Disease Incidence**	Mortality (%)	Plant survival time (days)	Nematodes per gram of fresh tissue
WFNS*	14	12	0.55	85.7	55.8 (42-69) a***	1095.7 (2.0-4013.2) a
WFNSM	14	6	0.43	57.1	61.0 (42-88) a	226.2 (5.7-826.6) a
BFNS	11	11	0.63	100.0	51.3 (42-88) a	1189.0 (2-5786.8) a
CK	15	0	0	0		

* WFNS: nematodes isolated from wood logs, cultured on *B. cinerea* for one cycle and non-surface sterilised before inoculation; WFNSM: nematodes isolated from wood logs, cultured on *B. cinerea* for several cycles and non-surface sterilised before inoculation; BFNS: nematodes isolated from *Monochamus* adults, cultured on *Botrytis cinerea*, but non-surface sterilised before inoculation; CK: untreated control.
** Disease incidence calculated 42 days after plants inoculated nematodes at 20th June, 2006.
*** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test ($P<0.05$).

6.3.1.2 Natural inoculation

In the 2004 experiment with 3-4-year old seedlings, 15 beetles were divided over three groups of five plants for a 1-, 2- or 3-day feeding period on *P. thunbergii*. Six seedlings died after these periods. Four plants were killed by three female and one male beetle after three days of feeding. One seedling died after one day's feeding by one female; another one died after two day's feeding by a female (Table 6.10).

Compared to the artificially inoculated seedlings, wilt development on naturally inoculated plants was slower (disease incidence: 0.32 *vs.* >0.47) and plant survival was lower (46.2 % *vs.* >71.4%). In the experiment with 7-9-year old plants, only one plant died 83 days after one female had been feeding for one day. All beetles were still carrying dispersal juveniles after the inoculation tests.

Table 6.10 Effect of natural inoculation tests using *Monochamus alternatus* carrying *Bursaphelenchus xylophilus* on 3-4 years old seedlings of *Pinus thunbergii* in 2004 (5 beetles for each treatment).

Feeding time (days)	Plants killed	Average age of beetle (days after emergence from log)	Plant survival time (days)	Nematodes per gram of fresh tissue
1	1	19	66	3920.9
2	1	22	49	384.7
3	4	21.2	65.5 (55-83)	744.3 (89.0-4859.3)

In 2005, no wilt symptoms were observed on 3-4-year old *P. thunbergii* when 66 seedlings had been exposed individually to one beetle for one day. Twenty-seven out of the 66 beetles were carrying nematodes after feeding. The average number of nematodes retained in the beetles was 1820.4 (range: 50-8348). Of these 27 beetles, 12 carried between 1000 and 10,000 nematodes, 13 between 100-1000 nematodes and 2 had 1 to 100 nematodes.

6.3.1.3 Comparison of artificial with natural inoculation

The effect of artificial and natural inoculation methods on 3-4-year old seedlings (Table 6.11) and 7-9-year old plants (Table 6.12) was compared using the data obtained in the 2004 experiments irrespective the inoculum densities. The inoculations included (i) B: beetles as natural inoculation source, (ii) BDJ: dispersal juveniles extracted from beetles and inoculated as such, (iii) BFS: dispersal juveniles cultured on *B. cinerea* for a single cycle and surface sterilized, (iv) BFNS: dispersal juveniles cultured on *B. cinerea* for a single cycle without surface sterilizing.

On 3-4-year old seedlings, significant differences between the treatments were recorded in both plant survival (F = 15.27, df = 3, P < 0.0001) and number of nematodes recovered from dead plants (F = 5.529, df = 3, P = 0.001) (Table 6.11).

Table 6.11 Comparison of artificial and natural inoculation of *Bursaphelenchus xylophilus* on 3-4-year old seedlings of *Pinus thunbergii*. Nematodes were all originating from beetles but received different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Plant survival time (days)	Nematodes per gram of fresh tissue
B*	15	6	62.8 (49-83.0) a**	1097.2 (2.0-4859) a
BDJ	46	39	56.5 (40-76) b	474.9 (0-6082.7) b
BFS	14	10	61.0 (47-83) a	374.8 (15.1-1848.1) b
BFNS	14	14	50.6 (44-60) c	302.0 (0-1343.4) b

* B: natural inoculation by beetle; BDJ: nematodes in dispersal stage collected from adult *Monochamus*; BFS: nematodes cultured on *Botrytis cinerea* and surface sterilised before inoculation; BFNS: nematodes cultured on *Botrytis cinerea* but not surface sterilised; CK: untreated control.
** With a column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

On 7-9-year old plants, significant differences between treatments were recorded for the plant survival time (F = 12.79, df = 3, P < 0.001) but not in number of nematodes recovered from dead tissues (F = 1.3077, df = 3 P = 0.276) (Table 6.12).

Table 6.12 Comparison of artificial and natural inoculation of *Bursaphelenchus xylophilus* on 7-9-year old plants of *Pinus thunbergii*. Nematodes were all originating from beetles but received different treatments prior to inoculation.

Treatment	Plants tested	Plants killed	Plant survival time (days)	Nematodes per gram of fresh tissue
B*	15	1	83 a**	1414.5 a
BDJ	13	11	53.4 (49-66) b	1089.7 (47.3-3035.8) a
BFS	13	11	62.6 (49-83) c	1494.8 (95.8-9417.9) a
BFNS	14	13	61.5 (49-83) c	1494.8 (95.8-9417.8) a

* B: natural inoculation by beetle; BDJ: nematodes in dispersal stage collected from adult *Monochamus*; BFS: nematodes cultured on *Botrytis cinerea* and surface sterilised before inoculation; BFNS: nematodes cultured on *Botrytis cinerea* but not surface sterilised; CK: untreated control.
** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

6.3.2 Pathogenicity tests of *Bursaphelenchus xylophilus* populations on *Pinus thunbergii* and three other *Pinus* species

6.3.2.1 Effect on three to four-year old seedlings of *Pinus thunbergii*

In 2005, 200 *B. cinerea*-cultured nematodes without surface sterilization from each of five populations of *B. xylophilus* were artificially inoculated onto 3-4-year old seedlings of *P. thunbergii* by bark-cutting (see 6.2.1.2). The symptom development was observed at

two-week intervals and the disease incidences were calculated six weeks after inoculation. The mortality of plants and the RHS index were calculated and listed in Table 6.13.

Inoculations with population BxCAN caused slower symptom development and lower mortality when compared to the other four populations (BxLYG, BxSD, BxJAP and BxPOT). Between the nematode populations, significant differences were recorded in their effect on plant survival time ($F = 2.99$, $df = 4$, $P = 0.029$) and number of nematodes extracted from dead plant tissue ($F = 6.204$, $df = 4$, $P = 0.0005$) (Table 6.13).

Table 6.13 Pathogenicity tests with different populations of *Bursaphelenchus xylophilus* inoculated artificially on *Pinus thunbergii* 3-4-year seedlings in 2005.

Population	Plants tested	Plants killed	Disease incidence**	Mortality (%)	RHS ***	Plant survival time (days)	Nematodes per gram of fresh tissue
BxLYG*	14	14	0.72	100.0	298.5	57.1 a (41-75) ****	2984.6 ab (35.6-7677.3)
BxSD	15	9	0.6	60	39.3	52.3 a (41-75)	655.6 a (27.3-2077.9)
BxCAN	14	2	0.31	14.3	0.8	65 a (45-75)	57.2 a (8-86.7)
BxJAP	14	13	0.86	92.9	80.0	57 a (41-75)	861.8 a (147.5-2304.5)
BxPOT	15	14	0.83	93.3	393.3	42.3 b (29-69)	4213.7 b (260.2-9418.6)
CK	15	0	0	0			

* Code see table 6.1.
** Disease incidence calculated 42 days after plants inoculated nematodes at 17th June, 2005.
*** RHS: relative host suitability index.
**** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test ($P<0.05$).

The repetition of the pathogenicity tests with the five *B. xylophilus* populations on *P. thunbergii* seedlings in 2006 and 2007 revealed similar patterns of symptom development and mortality of plants as obtained in 2005. Inoculations with population BxCAN again caused slowest symptom development. Inoculations with populations BxSD and BxJAP showed a similar speed of symptom development, while inoculations with BxLYG and BxPOT showed rapid wilt symptom development (e.g. Fig 6.2 for 2007).

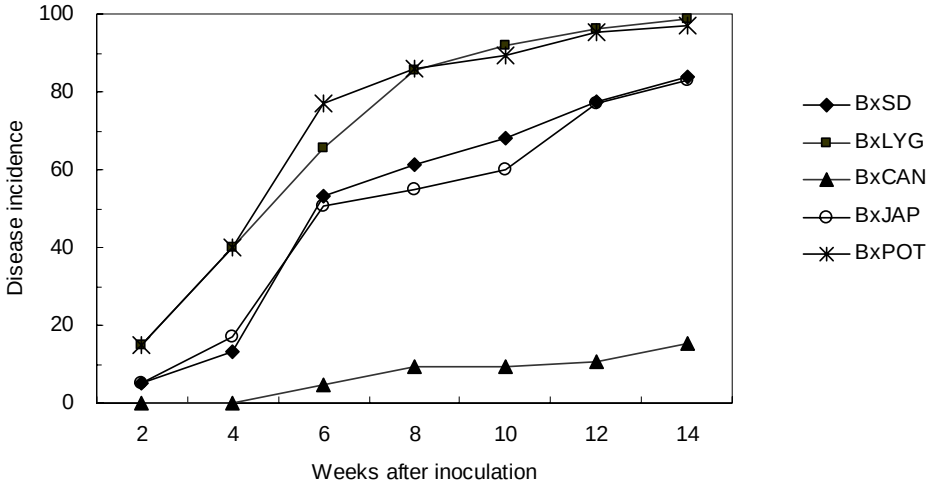

Fig 6.2 Symptom developments (expressed as disease incidence) caused by artificial inoculation of 200 *Botrytis cinerea*-cultured nematodes without surface sterilisation per plant from five populations of *Bursaphelenchus xylophilus* (see table 6.1) on 3-4-year-old seedlings of *Pinus thunbergii*. Disease incidence is calculated at 6 weeks after plants inoculated nematodes at 19th June, 2007.

In 2006, there were significant differences in plant survival time ($F = 10.47$, $df = 4$, $P < 0.0001$) between the 5 populations of *B. xylophilus*. There was no significant difference in the number of nematodes extracted from dead plant tissue inoculated with any of the five populations except BxCAN ($F = 0.605$, $df = 4$, $P = 0.662$) (Table 6.14).

Table 6.14 Pathogenicity tests with different populations of *Bursaphelenchus xylophilus* inoculated artificially on *Pinus thunbergii* 3-4-year seedlings in 2006.

Population	Plants tested	Plants killed	Disease incidence **	Mortality (%)	RHS ***	Plant survival time (days)	Nematodes per gram of fresh tissue
BxLYG*	15	15	0.56	100.0	135.7	57.5 a**** (42-88)	1356.9 a (55.5-7520.9)
BxSD	14	13	0.47	92.9	235.7	65.9 a (42-69)	2536.6 a (264.3-12558.8)
BxCAN	14	1	0.13	7.1	0	0 b	0
BxJAP	12	9	0.49	75.0	146.9	68.9 a (56-88)	1958.1 a (111.8-10342)
BxPOT	12	12	0.68	100.0	61.2	53.3 a 42-69)	612.1 a (214.7-12558.8)
CK	15	0	0	0			

* Code see table 6.1.
** Disease incidence calculated 42 days after plants inoculated nematodes at 20th June, 2006.
*** RHS: relative host suitability index.
**** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test ($P<0.05$).

In 2007, there were no significant differences in plant survival ($F = 2.05$, $df = 4$, $P = 0.1045$) and numbers of nematodes extracted from dead plant tissue ($F = 1.557$, $df = 4$, $P = 0.2029$) between inoculations with different populations of *B. xylophilus* (Table 6.15).

Table 6.15 Pathogenicity tests with different populations of *Bursaphelenchus xylophilus* inoculated artificially on *Pinus thunbergii* 3-4-year seedlings in 2007.

Population	Plants tested	Plants killed	Disease incidence **	Mortality (%)	RHS ***	Plant survival time (days)	Nematodes per gram of fresh tissue
BxLYG*	15	15	65.3	100	64.8	94.4 a**** (68-140)	648.1 a (16.8-1196.9)
BxSD	15	12	53.3	80.0	125.0	101.5 a (68-140)	1562.1 a (48.0-3732.2)
BxCAN	15	1	4.6	6.7	6.4	140 a	955.7 a
BxJAP	13	9	50.8	69.2	92.0	102.7 a (68-140)	1329.2 a (40.4-4013.5)
BxPOT	13	12	76.9	92.3	94.6	81.5 a (68-140)	1024.6 a (69.3-3518.5)
CK	15	0	0	0			

* Code see table 6.1.
** Disease incidence calculated 42 days after plants inoculated nematodes at 19th June, 2007.
*** RHS: relative host suitability index.
**** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

6.3.2.2 Effect on seven to nine-year old plants

In 2006, 200 nematodes from each of the five populations of *B. xylophilus* were inoculated onto 7-9-year old plants of *P. thunbergii*. Inoculations with the population BxCAN again showed slower symptom development and lower mortality when compared to inoculations with the other four populations (Table 6.16).

Table 6.16 Pathogenicity tests with different populations of *Bursaphelenchus xylophilus* inoculated artificially on *Pinus thunbergii* 7-9-year old plants in 2006.

Population	Plants tested	Plants killed	Disease Incidence **	Mortality (%)	RHS ***	Plant survival time (days)	Nematodes per gram of fresh tissue
BxLYG *	14	6	0.43	57.1	12.9	61 a**** (42-88)	226.2 a (5.7-826.6)
BxSD	14	7	0.28	50.0	369.8	81.7 a (69-88)	7395.4 a (73-16786)
BxCAN	15	2	0.05	13.3	1.2	72 a (56-88)	90.8 a (10.5-171.1)
BxJAP	14	7	0.17	50.0	147.4	75.3 a (56-88)	2948.9 a (2.0-14370)
BxPOT	14	6	0.36	42.9	65.6	49 b (42-56)	1529.8 a (45.7-4924.3)
CK	15	0	0	0			

* Code see table 6.1.
** Disease incidence calculated 42 days after plants inoculated nematodes at 20th June, 2006.
*** RHS: relative host suitability index.
**** With each column, means followed by the same letter are not significantly different from each other according to Tukey Test (P<0.05).

The RHS value obtained after BxCAN-inoculations was lower than that obtained for the other four populations. Between the nematode populations, significant differences were recorded in plant survival time (F = 3.237, df = 4, P = 0.038) but not in the number of nematodes extracted from dead plant tissue (F = 1.5802, df = 4, P = 0.2249) (Table 6.16).

6.3.2.3 Seedlings of three other *Pinus* species

In 2006 and 2007, the susceptibility of three other pine species, *viz. P. massoniana*, *P. taeda* and *P. elliotti* was compared to the susceptibility of *P. thunbergii* to the five populations of *B. xylophilus*. Each seedling was inoculated with 200 *B. cinerea* cultured nematodes without prior surface sterilisation and symptom development was observed at two-week intervals. The data on the pathogenicity of the nematode populations on *P. thunbergii* was analyzed in 6.3.2.1 and 6.3.2.2. During the two years of observations, no plants of *P. massoniana*, *P. taeda* and *P. elliotti* were killed after nematode inoculation. Some seedlings of *P. massoniana* showed green-yellowish needles two months after inoculation, but the plants did not die before the end of the observations. The susceptibility of the four *Pinus* spp. to five populations of *B. xylophilus* is summarised in Table 6.17, which is based on the results obtained from inoculation tests in 2006 and 2007.

Table 6.17 Pathogenicity of five populations of *Bursaphelenchus xylophilus* on 3-4 year old seedlings of four *Pinus* species as observed after artificial inoculation.

Population	*Pinus* species			
	P. thunbergii	*P. massoniana*	*P. taeda*	*P. elliotti*
BxLYG	+++**	+	-	-
BxSD	++	+	-	-
BxCAN	+	+	-	-
BxJAP	++	+	-	-
BxPOT	+++	+	-	-

* Code see table 6.1.
** +++: highly susceptible; ++: medium susceptible; +: low susceptible; -: resistant.

6.4 Discussion

The most promising measure for PWN control is the use of resistant or tolerant coniferous species. Nematologists define resistance as the ability of a plant to inhibit or prevent plant-parasitic nematodes from multiplying on that plant. Tolerance refers to the ability of the plant to withstand plant-parasitic nematodes by showing a normal plant growth and yield (Trudgill, 1991). Screening for both characteristics is based on the aggressiveness

and pathogenicity of PWN to plant species or varieties. Since *B. xylophilus* was confirmed as the causal agent of pine wilt disease (Kiyohara & Tokushige, 1971), investigations into the pathogenicity of *B. xylophilus* to different *Pinus* species have been carried out worldwide. The variations in results obtained from these pathogenicity tests were criticized (McNamara, 2004). Therefore different inoculation techniques were compared in this research evaluating the factors that affect the pathogenicity of *B. xylophilus* to *Pinus* species.

Natural inoculation using feeding beetles was examined to simulate the transmission of nematodes to plants in natural systems (Mamiya & Enda, 1972). Because the exodus of dispersal juveniles from the beetle occurs mainly between ten and 40 days after beetle emergence (Togashi & Sekizuka, 1982), the beetles were fed on fresh pine twigs for two weeks prior to their use. In 2004, beetles carrying a large amount of nematodes (Zhang *et al.*, 1995) were used to inoculate plants. Six out of 15 seedlings of *P. thunbergii* had died one, two or three days after their exposure to the beetles. Only one out of 15 7-9-year old plants had died after one day of beetle feeding. In 2005 none of the plants died although 27 out of the 66 beetles carried nematodes. The results of these experiments confirm that pine wilt disease can be induced by *B. xylophilus* transmitted by beetles feeding. However, the transmission is not assured.

Many factors affect the occurrence of pine wilt disease (Shibata & Okuda, 1989), the transmission efficiency of nematodes by beetles is one of the major factors (Jikumaru & Togashi, 2001). During my observations (chapter 5), the number of nematodes transmitted to plants by one beetle in one day varied between one and 350 individuals whilst beetles did not transmit nematodes every day. As a consequence, the initial number of nematodes loaded per beetle after emergence and the number of nematodes transmitted to feeding wounds during a certain time are impossible to determine precisely. The variable results obtained in the two years of natural inoculation tests demonstrated the problems of using nematode-carrying beetles as an inoculation tool in pathogenicity tests. Therefore, inoculations using beetles were not considered useful.

In the four years of artificial inoculation tests, different factors that may affect the pathogenicity of *B. xylophilus* were considered when designing the experiments, which included different (i) plant ages (3-4-year-old seedlings and 7-9-year-old plants), (ii) origins of nematodes (isolated from beetles or wood logs), nematode resources (dispersal juveniles from beetles or nematodes from fungal culture), culturing frequency (single or multiply generations), nematode contaminations (*i.e.* presence of symbiotic or associated bacteria) (surface-sterilized, heat-killed or alive non-sterilized) and nematode densities for inoculation.

In most previous PWN-pathogenicity research, thousands of *B. xylophilus* cultured on *B. cinerea* were used as inoculum on seedlings; the results of these experiments showed great variation (McNamara, 2004). To evaluate the effect of nematode density on the pathogenicity of *B. xylophilus*, different density levels were considered on the basis of my previous observations. In chapter 5, I demonstrated that the maximum number of nematodes transmitted to twigs by one beetle during the whole feeding period was 1638. Different densities of dispersal juveniles developed different dynamics in symptom expression and mortality of seedlings. The experiments demonstrated that both plant survival time and number of nematodes recovered from dead tissues depend on the nematode inoculation density. When plants were inoculated with PWN densities lower than 200 per tree, inoculations failed to cause any plant death (seedling tests in 2005). These small numbers of nematodes may have been trapped in the resinous surface of the wound and therefore may not have been able to survive. I therefore conclude that the number of nematodes inoculated onto plants has an important impact on assessing the pathogenicity of *B. xylophilus.*

In my study, using different numbers of dispersal juveniles extracted directly from beetles for inoculation caused a high percentage of plant death, which demonstrates that most reported pathogenicity tests using artificial inoculation methods overestimate the pathogenicity of *B. xylophilus* (Kiyohara & Tokushige, 1971; Wingfield *et al*., 1984; Dwinell, 1985; Melakeberhan & Webster, 1990). A density of 200 nematodes per tree is suggested as the appropriate number of nematodes in inoculations. This number is close to the maximum number (350) of PWN transmitted by one beetle in one day under natural conditions (chapter 5).

PWN with different contamination status showed clear differences in disease development after their inoculation. In 2004, the 3-4–year old seedlings of *P. thunbergii* inoculated with 1000 fungus-cultured nematodes without prior surface sterilisation (BFNS) developed faster symptoms and caused higher plant mortality compared to treatments with nematodes that were surface sterilised (BFS) or inoculation with dispersal juveniles (BDJ). However, 7-9-year old plants inoculated with 1000 BDJ developed faster wilting symptoms than plants inoculated with BFS and BFNS nematodes. In 2005, inoculations of 3-4-year old seedlings with 200 non-sterilised nematodes (BFNS) again provoked faster wilting and higher mortality than inoculations with sterilised nematodes (BFS). However, the 7-9-year old plants inoculated with 200 BFNS demonstrated similar symptom development and mortality as those inoculated with BFS. Obviously, lack of surface sterilisation prior to inoculation increases the pathogenicity of the PWN when inoculated at 1000 or 200 individuals per tree.

This suggests that the existence of microorganisms on the nematode body may increase the sensitivity of plants to the nematodes. Nevertheless, surface sterilized nematodes caused high mortality whereas heat-killed nematodes did not kill any plant. This suggests that *B. xylophilus* is the main agent causing pine wilt disease as opposed to any bacterial symbiont or associate. Both the inoculations with 1000 nematodes (in 2004) or 200 nematodes (in 2005) on 3-4-year old seedlings and 7-9-year old plants caused significant differences in plant survival time between different nematode treatments. Obviously seedlings are more sensitive to PWN inoculations than mature plants. Kishi (1999) also mentioned that wilt occurred more rapidly and at higher rates in young trees than in older ones.

To evaluate the effect of nematode resources and culture status on pathogenicity of *B. xylophilus*, both young seedlings and older plants were inoculated with nematodes collected from wood logs or beetles and cultured on *B. cinerea* during a single or multiple cycles and exposed to different treatments prior to inoculation. In 2006, 3-4-year old seedlings as well as 7-9-year old plants inoculated with nematodes collected from beetles and cultured for a single cycle on *B. cinerea* (BFNS) showed faster wilting compared to those inoculated with nematodes collected from wood logs and cultured for a single cycle (WFNS) or multiple cycles (WFNSM). However, in the experiment of 2007, 3-4-year old seedlings inoculated with BFNSM (nematodes collected from beetles followed by multiple cultures on *B. cinerea*) showed slower symptom development and lower mortality compared to WFNS, WFNSM and WFS (nematodes isolated from wood logs and cultured during a single cycle on *B. cinerea* followed by surface sterilisation). No WHK (nematodes collected from wood and cultured on fungus followed by heat-killing) inoculated seedlings died. The fact that dispersal juveniles cultured on fungus for multiple cycles (BFNSM) show decreased levels of pathogenicity compared to the one cycle fungus cultured nematodes (BFNS) suggests that chemical cues provided by beetle vectors may affect the pathogenicity of *B. xylophilus*. The lack of differences in plant survival time and number of nematodes isolated from wood between WFS, WFNS and WFNSM inoculations may lead to the conclusion that the sub-culturing for many generations on *B. cinerea* does not affect the rate of propagation of the nematodes, which relates to the pathogenicity of *B. xylophilus*. However, Kiyohara (1976) reported that *B. xylophilus* sub-cultured for five years on *B. cinerea* showed decreased pathogenicity to pines, resulting in a lower rate of propagation of the nematodes.

To study the variation within pathogenicity genes of *B. xylophilus* (see chapter 7), nematodes from five different populations were artificially inoculated onto *P. thunbergii*. In the 2005, 2006 and 2007 experiments, I obtained the same results on 3-4-year seedlings and

7-9-year old plants, *viz.* plants inoculated with population BxCAN always demonstrated slower symptom development and lower mortality when compared to the other four populations. Symptoms developed faster on the seedlings inoculated with populations BxLYG and BxPOT than on those inoculated with BxSD and BxJAP, which showed a comparable speed of symptom development. The RHS, which takes into account mainly the plant reaction related to the symptoms and the number of re-isolated nematodes in plants successfully inoculated, gives good indications on host susceptibility (Braasch, 1997). The RHS value obtained after BxCAN-inoculations was lower than that obtained for the other four populations, and demonstrates the lower propagation speed and lower pathogenicity of BxCAN on *P. thunbergii* (Caroppo *et al*., 2000; Skarmoutsos & Michalopoulos-Skarmoutsos, 2000).

Based on differences in disease incidence, mortality of plants and RHS index, three pathogenic groups of five *B. xylophilus* populations can be categorized: (i) highly pathogenic populations (e.g. BxLYG and BxPOT) causing rapid symptom development on susceptible plants and showing higher plant mortality and speed of nematode multiplication; (ii) medium pathogenic populations (e.g. BxSD and BxJAP) with medium speed of symptom development and slow multiplication speed on susceptible plants; (iii) populations with low pathogenicity (e.g. BxCAN) causing slow disease development on susceptible plants and low death rate and RHS value.

Two years of pathogenicity experiments with five *B. xylophilus* populations on three other pine species, *viz. P. massoniana*, *P. taeda* and *P. elliotti* revealed *P. massoniana* to be the least susceptible pine species to *B. xylophilus* and confirmed earlier Chinese studies (Bai & Cheng, 1993; Liu & Feng, 1994; Xu *et al*., 1996; Wang *et al.*, 1997). My observations classified *P. taeda* and *P. elliotti* as completely resistant, which confirms results obtained in previous research (Furuno, 1982; Xu *et al*., 1996; Li & Wang, 1997; Xu *et al*., 1998; Yang *et al.*, 2002), but contrasts with earlier reports (Dwinell, 1985; Yang *et al.*, 1987; Ge & Cheng; 1993, Yang & Wang, 1989) which classified *P. elliotti* as a susceptible or moderately susceptible species and *P. taeda* as a moderately susceptible or resistant species. Variation in results produced by pathogenicity tests has been criticized by McNamara (2004). Differences in nematode isolates used, age of plants, and pre-treatments are probably the cause of the variation in results.

Artificial inoculation experiments evaluating the pathogenicity of *Bursaphelenchus* species to *Pinus* species should consider different factors which may affect the results obtained; these include plant age, number of nematodes inoculated, origin of nematodes,

nematode resources, culturing frequency and nematode contamination. The environmental conditions should equally be considered; they include the experimental area (greenhouse or natural field), climate conditions (temperature and humidity), and the inoculation time because temperature affects the invasion of nematodes. The incidence of pine wilt disease requires a minimum summer temperature of 20°C (Rutherford *et al*., 1990). Very small nematode densities inoculated at sub-optimal periods (*e.g.* autumn) cause latent infections without symptoms in the inoculation year, but causing plant death in next year (Yang *et al*., 2002). In view of the results obtained in the four year pathogenicity experiments, I conclude that good results can be obtained with artificial inoculations of 200 *B. cinerea* cultured and non-surface sterilized nematodes on 3-4-year old seedlings in the early summer; at that time the average air temperature is higher than 20°C and the time matches the period of beetles' maturation feeding.

Chapter 7

Cloning and characterization of pathogenicity related genes from Bursaphelenchus xylophilus*

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Kikuchi, T., **Li, H.**, Moens, M. & Jones, J. (2008). Identification of Expansin-like genes from the pine wood nematode *Bursaphelenchus xylophilus* and evolution of the expansin gene family within the Nematoda. Submitted.

7.1. Introduction

The expression and expansion of pine wilt disease (PWD) depend on a range of biological and physical factors. The causal agent of PWD, *Bursaphelenchus xylophilus* displays wide variation in pathogenicity (Kiyohara & Bolla, 1990; Sutherland *et al*., 1991) and is associated with various host species across a wide geographical distribution (Ryss *et al*., 2005). The huge economic losses caused by this nematode require an understanding of the relationship between host and parasites, which is essential for designing disease control programs. The proteins secreted by parasitic nematodes play important roles in host-parasite interactions. Understanding the structure and function of secreted proteins encoded by nematode parasitism genes is necessary for disease control.

Although a variety of genes encoding proteins important in the host parasite interaction have been identified in endoparasitic root-knot nematodes and cyst nematodes, little was known about the molecular basis of host–parasite interactions in *B. xylophilus* populations. An expressed sequence tag (EST) project on *B. xylophilus* (Kikuchi *et al.*, 2007) has allowed the cloning and functional characterization of cellulase (endo-β-1,4-glucanase), β-1,3-glucanases and pectate lyase genes from *B. xylophilus* (Kikuchi *et al*., 2004; 2005; 2006). The expansin-like proteins, which are thought to disrupt non-covalent bonds between cellulose microfibrils in plant cell walls, have been identified in cyst nematodes (Qin *et al*., 2004; Kudla *et al*., 2005), but not been identified in *B. xylophilus* yet. No pathogenicity associated proteins have been identified in nematodes to date.

RNAi has been used to identify essential genes of root-knot nematodes and cyst nematode that may serve as novel control targets for transgenic resistance. No RNAi studies have been reported for any migratory parasitic nematodes. Therefore, the aims of the work undertaken in this chapter were:

1. To analyse the sequences of selected *B. xylophilus* pathogenicity genes in a range of nematode populations that differ in their pathogenicity in order to attempt to identify differences in sequences that may be correlated with differences in nematode pathogenicity.
2. To characterize selected pathogenicity genes using *in situ* hybridization.
3. To develop a method for RNAi in *B. xylophilus* and to attempt to apply this method for functional analysis of genes expressed in the nematode gland cells.

7.2 Materials and methods

7.2.1 Analysis of variation in four pathogenicity related genes in populations of *Bursaphelenchus xylophilus* showing different pathogenicity characteristics

7.2.1.1 Nematode populations

Five populations of *B. xylophilus* were selected for a range of pathogenicity characteristics according to results of the pathogenicity tests carried out in during 2005 to 2006 (see Chapter 6). The origin of the nematodes and their host plants are seen in Table 6.1. The nematodes were maintained on fungal cultures of *Botrytis cinerea* and extracted with a modified Baermann funnel and cleaned twice with SDW (see chapter 3).

7.2.1.2 Genomic DNA extraction

Genomic DNA was extracted from up to 100μl of *B. xylophilus*. The nematode pellet was ground in liquid nitrogen with a mortar and pestle until a fine powder was obtained, 250μl 2× DNA extraction buffer (200mM Tris pH8.5, 200mM NaCl, 50mM EDTA) was added to the powder which was allowed to defrost. The liquid was transferred into a 2ml tube and the mortar was rinsed with a further 250μl 2× DNA extraction buffer and the liquid added to the same tube. After adding 350μl SDW, 50μl 20mg/ml Protease K and 100μl 10% SDS, the mixture was incubated for 1 hour at 60°C to release the DNA from cells. One ml of Phenol/Chloroform/Isoamyl alcohol was added to the tube. The tube was mixed very gently and centrifuged for 5min at maximum speed (13,000 × *g*) in a micro centrifuge. The top layer was transferred to a fresh tube and 1ml Chloroform/Isoamyl alcohol was added. The tube was centrifuged as above and the top layer was removed to a fresh tube. 20μl RNase A (10mg/ml) was added and the mixture was incubated at 37°C for 30 min. The Phenol/Chloroform/Isoamyl alcohol extraction steps described above were repeated once. 1/10 volume 3M Sodium Acetate and 2.5 volumes 100% ethanol were added to the final aqueous layer and the mixture was incubated at -20°C for at least 30 min to precipitate the DNA. A DNA pellet was formed after centrifuging at top speed (13,000 × *g*) for 15 min and this was rinsed briefly with 250μl 70% ethanol followed by centrifuging for 2 min at top speed. After removing the ethanol, the pellet was air-dried and resuspended in an appropriate volume of water and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA).

7.2.1.3 Total RNA extraction (Trizol method)

Total RNA was extracted from a 50μl pellet of *Bursaphelenchus* spp. Nematodes were ground in liquid nitrogen in a mortar and pestle until powder and 1 ml of Trizol reagent (Sigma) was added. The liquid was placed into a fresh tube and homogenized 20 times through a 20 gauge syringe needle. The mixture was incubated for 5 min at room temperature (RT) and then 200μl chloroform was added, the mixture was shaken for 15 seconds and then incubated at RT for 2-3 min. After being centrifuged at 12,000 × *g* for 15 min at 4°C, the upper aqueous phase was removed into a fresh tube and 500μl isopropanol was added and the tube was inverted several times. The mixture was incubated for 15 min at room temperature and then centrifuged at 12,000 × *g* for 10 min at 4°C to allow the RNA pellet to form. After removing the supernatant, 1ml 75% ethanol was added and the tube was centrifuged at 7,500 × *g* for 5 min at 4°C. Subsequently, the supernatant was discarded and the tube was air dried for 10 min. The RNA pellet was resuspended in 30-50μl RNase free water and incubated for 10 min at 55-60°C to allow the pellet to dissolve completely. RNA was stored at -80°C or used directly for cDNA synthesis.

7.2.1.4 mRNA extraction (Dynabeads method)

Messenger RNA was prepared using a Dynabeads mRNA DIRECT^{TM} Micro Kit according to the manufacturer's instructions (DYNAL A.S., Oslo, Norway). The Dynabeads Oligo $(dT)_{25}$ stock was resuspended thoroughly and the amount needed for all samples (20μl per sample) was transferred into an RNAase-free 1.5 ml tube. The tube was placed in a magnetic particle concentrator (Dynal MPC) for 30 sec until the suspension was clear. After removing the supernatant, the Dynabeads were prewashed once by resuspending in Lysis/Binding Buffer, concentrating on the magnetic stand as described above and finally resuspended in 20μl of Lysis/Binding Buffer per sample.

A 30-50μl pellet of nematodes was ground in liquid nitrogen until powder with a pestle in a 1.5 ml tube and 100μl Lysis/Binding Buffer was added. After adding 20μl of the prewashed Dynabeads Oligo $(dT)_{25}$ to the tube, the mixture was rotated for 5 min at room temperature. The tube was then placed on the magnet, the supernatant removed and 100μl Washing Buffer A was added. These steps were repeated once to allow another wash of the beads with Washing buffer A. The Dynabeads-mRNA complex was then rinsed twice in 100μl Washing Buffer B using the magnetic stand procedure described above and the

mixture was transferred to a new tube. Finally the Dynabeads-mRNA complex was resuspended in 50μl ice-cold 10mM Tris-HCl. This mixture was stored at -20°C or used directly for cDNA synthesis.

7.2.1.5 Complementary-DNA (cDNA) synthesis

First strand cDNA was synthesized using the SuperScript III first stand synthesis system for RT-PCR (Invitrogen, UK) according to the manufacturer's recommendations. Eight microlitres of RNA template was combined with 1μl of 50μM oligo $(dT)_{20}$ primer and 1μl of 10mM dNTP mix and the mixture was incubated at 65°C for 5 min in order to denature RNA secondary structure. The mixture was then placed on ice for at least 1 minute.

The cDNA synthesis mix was prepared by mixing 2μl of 10× RT buffer, 4μl of 25mM $MgCl_2$, 2μl of 0.1 M DTT, 1μl of RNase OUT (40U/μl) and 1μl of SuperScript III RT (200 U/μl) for each cDNA synthesis reaction. 10μl of the resulting cDNA synthesis mix was added to each RNA/primer mixture. After mixing gently and centrifuging briefly, the mixture was incubated for 50 min at 50°C. The reaction was terminated by incubating at 85°C for 5 min and chilled on ice. cDNA synthesis products were stored at -20°C or used for PCR immediately.

7.2.1.6 PCR amplification

PCR primers listed in Table 7.1 were designed using primer design software and were synthesized by Sigma, UK.

Table 7.1 Primers used for pathogenicity related genes.

Gene	Code	Primer sequence (5'-3')	Tm
Actin	BxactinF	CGAGAAGTCCTATGAACTTC	55.4
	BxactinR	CACATCTGTTGGAAGGTGGAC	64.4
Cellulase	BxENG00F	TCTAAAATGAAGTCTCTTGTG	53.6
	BxENG00R	AGTCCTCTAAGCATCGTC	54.1
β-1,3-endoglucanase	Bx13engF	ATGAGAGTTGTCATTGCC	55.7
	Bx13engR	CACCGAAAACTACAACGT	56.0
Pectate lyase	BxpelF	GTCCGTTGAAGATGGTCCAA	64.9
	BxpelR	CAGATAATTAGGTTCAGAACG	55.5
Expansin	BxEXPF	GACCAGATCACTCCCCAGTTG	65.8
	BxEXPR	ACATCCGCTGGCAGGGCTAG	70.7
	BxEXPR1	ACAGCAGTTTTTCCCTTGTAC	59.7

The PCR mixture (25μl) contained 1× reaction buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl), 1.5mM $MgCl_2$, 200μM dNTPs, 1μM each primer, 1 unit *Taq* DNA polymerase (Promega) and 1μl DNA (cDNA or gDNA) template. Amplification was carried out using a Gene Amp R PCR system 9700 (Applied Biosystems) employing an initial denaturation step at 94°C for 2 min, 30 reaction cycles of denaturing 94°C for 30 sec, annealing 55°C for 30 sec (with the temperature adjusted depending on the Tm of each primer pair) and extension 72°C for 1 min, followed by a final extension at 72°C for 7 min. After PCR, 5μl of amplified product was analysed by electrophoresis in a 1% agarose gel (Sambrook *et al.*, 1989) (see chapter 3).

7.2.1.7 Cloning and Sequencing

PCR products run on a 1% agarose gel were purified and ligated into pGEM-T Easy vector (see chapter 3). The ligation was transformed into DH5 α competent cells (Invitrogen, UK) by electorporation using a MicroPulser (BIO-RAD). The plasmid DNA was extracted for sequencing (see chapter 3).

7.2.1.8 Comparing the variation of the genes between populations

DNA sequences obtained from plasmids were aligned using ClustalW in BioEdit and Phylogenetic analyses were performed in MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 (Kumar *et al*., 2004). The pairwise sequence divergences were calculated and the phylogenetic trees were generated by neighbour-joining (NJ) analysis (Saitou & Nei, 1987) using the P-distance option. The robustness of the trees was tested using the bootstrap method (Felsenstein, 1985). All bootstrap values are based on 1000 replicates and the cut off value for consensus tree is 50%.

7.2.2 Functional Analysis of Expansin genes of *Bursaphelenchus xylophilus* and *B. mucronatus* by *in-situ* Hybridization

In situ hybridization was performed as described by De Boer *et al.* (1998) with some modifications.

7.2.2.1 Preparation of DIG-labelled DNA probes

A gene encoding a protein similar to expansins was identified in ESTs of *B. xylophilus* (Kikuchi *et al*., 2007). A PCR primer pair BxEXPF and BxEXPR1 (Table 7.1) was designed

to amplify a 250 bp fragment of this gene from cDNA (or plasmid stocks) of *B. xylophilus* and *B. mucronatus*. This fragment was used to prepare DIG-labelled DNA probes for *in situ* hybridization. The PCR product was purified using a MinElute PCR purification Kit (Qiagen, UK) and run on a gel to check the quantity. Purified PCR product was used in a linear PCR reaction to incorporate the DIG labelled UTP. The PCR was carried out with the sense and antisense primer in separate reactions and consisted of a predenaturing step at 94°C for 2 min followed by 35 cycles of 94°C 15sec, 55°C 30 sec and 72°C 90 sec, and finally 72°C for 4 min. The probes were checked on a 1% agarose gel to confirm that a product of the desired length has been synthesised. Probes generated from the antisense strand of the PCR product should hybridise to the mRNA present in the nematode while those generated from sense strands should not hybridise and serve as a negative control.

7.2.2.2 Fixation and cutting of nematodes

Freshly harvested nematodes were concentrated to a 30-50μl pellet and resuspended in 1ml fixative (2% paraformaldehyde in M9 buffer). Nematodes were fixed for 18hr at 4°C and then for a further 4 hr at room temperature. The fixative was removed and the fixed nematodes were resuspended in 200μl of 10 × diluted fixative and then transferred onto a clean microscope slide. The nematodes were cut with a single edge razor blade taped to a vibrating aquarium air pump. The chopped nematodes were checked under a microscope to ensure all of them had been cut into approximately 2-4 pieces. The nematode pieces were then collected into a 1.5ml tube by rinsing off the slide in 10 × diluted fixative.

7.2.2.3 Permeabilization of nematodes

Nematode sections were washed twice in 1ml M9 buffer and then incubated in 0.5ml proteinase K solution (0.5mg/ml in M9 buffer) for 25-30 min at 22°C on a rotator. The nematodes were washed once with M9 buffer and then centrifuged to form a pellet which was placed at -20°C for 15 min. The nematodes were resuspended in one ml of cold (-20°C) methanol and incubated on deep frozen ice for 30 sec. The nematodes were centrifuged at 13,000 × *g* for 30 sec and the pellet was resuspended in 1ml of cold (-20°C) acetone and left for 1 min on ice. After centrifuging at 13,000 × *g* for 1 min, acetone was removed until approximately 100μl remained in the tube. The nematode pellet was then rehydrated by adding 100μl of DEPC-treated ddH2O drop by drop to this remaining acetone.

7.2.2.4 Hybridisation

During all the following incubations the nematodes were rotated in a 1.5ml RNase-free microfuge tube and all centrifuge steps were carried out at RT at 10,000 × *g* for 2 min. The permeabilised nematodes were centrifuged and resuspended in 500μl hybridization buffer. The nematode sections were then centrifuged and resuspended in fresh hybridization buffer, using 150μl buffer per hybridization, and distributed into 0.5ml tubes. The nematodes were then prehybridized for 15 min at 50°C. DIG-labelled probes were denatured at 95°C for 10 min and cooled rapidly on ice. The probes were then added to the hybridisation solution containing the nematode sections. The mixture was rotated overnight at 50°C to allow hybridization of the probes to mRNA targets. The nematodes were then washed three times for 15 min with 4× SSC at 50°C and three times for 20 min with 0.1× SSC/0.1% SDS at 50°C.

7.2.2.5 Staining

Nematodes were washed with 200μl maleic acid buffer for 1min and then incubated for 30 min in 200μl of 1% Boehringer Blocking reagent in maleic acid buffer. The nematodes were then incubated for 2 hr in 200μl 1% Boehringer Blocking reagent in maleic acid buffer containing 1:1000 diluted alkaline-phosphatase conjugated anti-digoxigenin antibody (Boehringer). Unbound antibody was removed by washing three times for 15 min in 200μl of maleic acid buffer containing 0.01% Tween-20. The nematodes were washed briefly in 200μl of alkaline phosphatase detection buffer and then stained in 200μl of Nitro Blue tetrazolium /X-phosphatase (Boehringer) staining solution for 16-14 hr at 4°C. The staining reaction was stopped by washing the nematodes twice in 0.01% Tween-20 in sterile H_2O.

7.2.2.6 Preparing slides

The nematodes were centrifuged and the supernatant was removed until approximately 50μl was left in the tube. The nematodes were resuspended with a pipette and 16-17μl was placed on a microscope slide. The nematode sample was then covered with a coverslip and sealed with nail vanish. The nematodes were examined with differential interference microscopy and photographs were taken using Olympus Model BxF camera.

7.2.3 Development of a method for RNAi in *Bursaphelenchus xylophilus*

7.2.3.1 Generation of dsRNA

The primer sequences used for dsRNA generation are listed in the Table 7.2.

Table 7.2 Primer codes used for dsRNA synthesis and their sequences.

Protein	Primer code	Primer sequence (5'-3')
RPS23	Rps23F	GAGAAGATCGGTGTCGAAGC
	Rps23F	AACAGGGCAATCAGGGAAGT
	TESTrps23F	CGCTGGCATGATCAGAGAT
	T7Rps23F	GTAATACGACTCACTATAGGGGAGAAGATCGGTGTCGAAGC
	T7Rps23F	GTAATACGACTCACTATAGGGAACAGGGCAATCAGGGAAAGT
β-1,3	13EGF	ACTTGACCACCGACTTCCAC
endoglucanase	13EGR	CCAACATCTGGCCTGGTAAT
	T713EGF	GTAATACGACTCACTATAGGGACTTGACCACCGACTTCCAC
	T713EGR	GTAATACGACTCACTATAGGGCCAACATCTGGCCTGGTAAT
	TEST13EGF	GAGAACGGCAACTTGGTGAT
	TEST13EGR	AAGGTGCCGAGGATCTCTTT
GFP	GFPF	GCTGGAGTACAACTACAACT
	T7GFPF	GTAATACGACTCACTATAGGGGCTGGAGTACAACTACAACT
	GFPR	GGCAGATTGCGTGGACAGGT
	T7GFPR	GTAATACGACTCACTATAGGGGGCAGATTGCGTGGACAGGT

Three genes were used in experiments in which a method for RNAi in *B. xylophilus* was developed; *β*-1,3-endoglucanse (1,3*eng)*, Ribosomal Protein Subunit 23 (*rps* 23) and Green Florescent Protein (*gfp)* as a negative control. A set of primers was designed in the C-terminus-encoding region for each of the genes. These primers were synthesized twice, one set with the T7 sequence incorporated at the 5' end of each primer and the other without the T7 sequence. Separate PCR reactions using these primers were performed in which a 250bp fragment of each of the genes were amplified with the T7 promoter sequence incorporated at the 5' end of either the sense or antisense strand.

A diagram summarizing the procedures used for synthesis of dsRNA is provided in Fig 7.1.

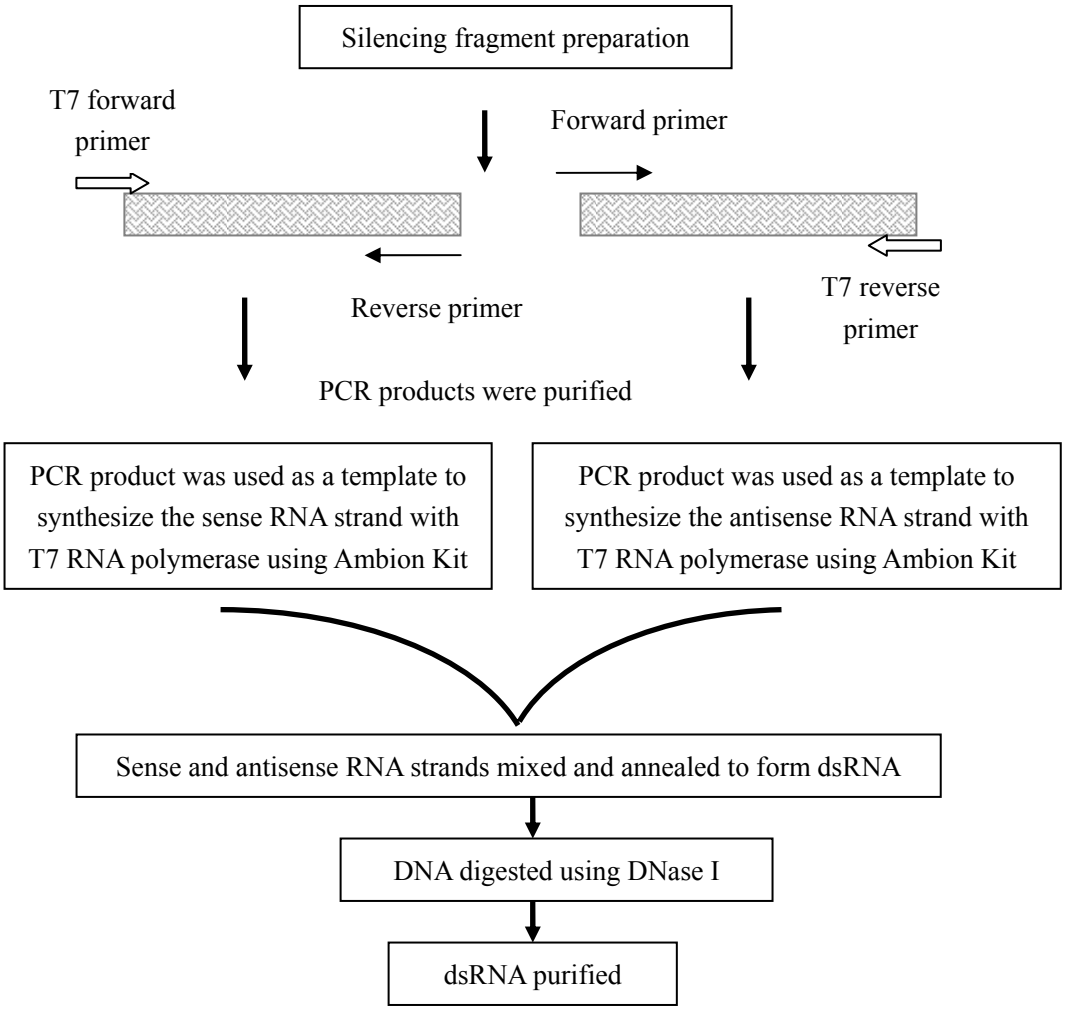

Fig 7.1 Schematic diagram of the dsRNA synthesis procedure.

The PCRs were carried out as described above and the product of each PCR was purified using a MinElute PCR purification Kit (Qiagen, UK)(see chapter 3). 1μg of each PCR product was used for transcription with T7 RNA polymerase in separate reactions using a MEGAscript RNAi Kit (Ambion, UK) as described by Chen *et al.* (2005). Briefly, in each 20μl reaction, 4μl of PCR product and 2μl of each of 10× T7 reaction buffer, ATP, CTP, GTP and UTP solutions and T7 Enzyme Mix were added to 0.5ml nuclease-free tubes. The reactions were incubated at 37°C for 16 hr. The reaction products were checked on a 1% agarose gel and then sense and antisense RNA strands were mixed together. The mixtures were heated at 75°C for 5 min then cooled to room temperature to allow the complementary strands to anneal. DNA template was removed in a DNA digestion reaction performed using 2μl DNase I and 5μl digestion buffer and incubated for 1 hour at 37°C. Finally the dsRNA

was purified using filter cartridges (Ambion, UK), precipitated under ethanol and eluted in 100μl of hot (>95°C) elution buffer. The quantity of dsRNA was measured using a Nanorop ND-1000 Spectrophotometer. For experiments examining uptake of dsRNA by *Bursaphelenchus* spp.. A dsRNA reaction was carried out in which UTP was replaced with Cy3 labelled UTP (Amersham) in order to generate fluorescently labelled dsRNA.

7.2.3.2 Analysis of uptake of dsRNA by *Bursaphelenchus* spp.

Experiments were performed to examine the uptake of dsRNA by *B. xylophilus* and *B. mucronatus* and the effects of the neurotransmitter Octopamine on the proportion of nematodes taking up dsRNA from solution. Freshly harvested *B. xylophilus* and *B. mucronatus* were cleaned twice with SDW and the nematodes were concentrated in 100μl SDW. Each nematode sample was divided into two tubes and 50μl Cy3 labelled dsRNA was added to each tube. Octopamine (at a final concentration of 50mM) was added to one tube for each species. The tubes were covered with aluminium foil and incubated at RT in a rotator for 24 hr. The nematodes were rinsed with SDW containing 0.1% Tween 20 three times, with a 4 min centrifuge at 8,000 × *g* between each rinse. 50 μl aliquots of each of the four samples were observed under light and fluorescence microscopes. First, the proportion of living nematodes in each sample was calculated. Then the numbers of live nematodes that had taken up labelled dsRNA into their digestive system was scored. In each case at least 100 individuals were scored.

7.2.3.3 RNAi by soaking

Freshly cultured *B. xylophilus* were collected from fungal plates and cleaned twice with SDW. The nematodes were concentrated to a 40-50μl pellet by centrifuging at 8000 × *g* and removing most of the liquid. 45μl of dsRNA at a concentration of 1.5 – 2 μg/μl (*rps*23, 1,3*eng* or *gfp*) was added to the nematodes along with 1μl 5% gelatin and 1μl 300mM spermidine. In later experiments the dsRNA was combined with 45μl lipofectin or lipofectamine (1mg/ml, Invitrogen) before being added to the nematodes. The tubes were covered with aluminium foil and incubated at RT in a rotator for 24 hr.

After soaking in dsRNA, the nematodes were washed three times with SDW and divided into two parts. One part was used for mRNA extraction, and the other part was used for phenotype analysis.

7.2.3.4 Quantitative RT-PCR experiments

mRNA was extracted using the Dynabeads mRNA DIRECT^{TM} Micro Kit and first strand cDNA was synthesized using the SuperScript III cDNA synthesis system as described above. The levels of the targeted mRNA and a control gene (Actin) were analysed by semi quantitative PCR. The PCR mixture (50μl) contained 1× reaction buffer, 1.5mM $MgCl_2$, 200μM dNTPs, 1μM of the relevant primer pair (Table 7.3), 1 unit *Taq* DNA polymerase (Promega, USA) and 1μl cDNA template. Amplification was carried out with a predenaturing at 94°C for 2 min, 35 reaction cycles of denaturing 94°C for 30 sec, annealing 55°C for 30 sec and extension 72°C for 1 min, followed by a final extension at 72°C for 7min.

Table 7.3 Primers used in RT-PCR.

Gene	Primer code	Primer sequence (5'-3')	Tm
*rps*23	TESTrps23F	CGCTGGCATGATCAGAGAT	64.1
	Rps23R	AACAGGGCAATCAGGGAAGT	64.6
1,3*eng*	TEST13EGF	GAGAACGGCAACTTGGTGAT	64.0
	TEST13EGR	AAGGTGCCGAGGATCTCTTT	63.9
Actin	BxactinF	CGAGAAGTCCTATGAACTTC	55.4
	BxactinR	CACATCTGTTGGAAGGTGGAC	64.4

In each RNAi experiment the effects of soaking in dsRNA from the target gene (*rps*23 or 1,3*eng*) were compared with the effects of soaking in the non-endogenous gene (*gfp*) as a negative control. Two cDNA samples were therefore generated in each experiment (one from nematodes soaked in *gfp* dsRNA and the other from nematodes soaked in *rps*23 or 1,3*eng* dsRNA) and each cDNA sample was used for two PCR reactions, one using actin primers and one using primers against the targeted gene. 10µl of the PCR reaction products were sampled from each PCR reaction after 20, 23, 26 and 30 cycles. All products were analysed by electrophoresis in a 1% agarose gel.

7.2.3.5 Examination of the effect of gene silencing on phenotypes

The two genes analysed were expected to show different phenotypes. For *rps*23 a lethal phenotype was expected. Therefore, after soaking in dsRNA and cleaning in SDW as described above, the nematodes were left in a large volume (1-2ml) SDW and the proportion of dead nematodes was calculated after 24h, 2d and 3d. The numbers of dead nematodes resulting from incubation in *gfp* and *rps*23 were compared. For counting, 50 μl aliquots of

nematode suspension were used and each count was replicated at least three times. For 1,3*eng* the expected phenotype was a reduced efficiency of feeding on fungi and therefore a reduced rate of nematode population build up. For these experiments nematodes soaked in dsRNA from the 1,3*eng* and *gfp* genes were counted and aliquots containing 100 nematodes were inoculated onto small plates of *B. cinerea*. The numbers of nematodes recovered from the plates using a modified Baermann funnel at 20°C for 24 hr were counted after 2 days and 5 days. At least 5 replicates for each treatment were carried out.

7.3 Results

7.3.1 Variation in pathogenicity related genes from populations of *Bursaphelenchus xylophilus* showing different pathogenicity characteristics

7.3.1.1 Amplification of pathogenicity related genes

Genomic DNA and total RNA were extracted from five nematode populations of *B. xylophilus* and the quantity and quality were assessed by spectrophotometry. The concentrations and A260/280 ratios for each sample are listed in Table 7.4. cDNA was synthesized from each total RNA sample and fragments of four pathogenicity related genes and one control gene (actin) were amplified with primer pairs specific to each gene (Table 7.1). Where it was not possible to obtain a product using PCR amplification from cDNA, genomic DNA was used as a template instead.

Table 7.4 Quantity and quality of genomic DNA and total RNA extracted from *Bursaphelenchus xylophilus* populations.

Population	Genomic DNA			Total RNA		
	ng/μl	260/280	260/230	ng/μl	260/280	260/230
BxSD	1001.8	2.29	1.39	3723.6	1.84	1.95
BxLYG	492.7	2.35	1.11	1656.2	2.06	1.69
BxCAN	843.9	2.35	1.14	2694.2	2.03	1.88
BxJAP	903.6	2.25	1.17	1510.4	2.07	1.38
BxPOT	944.8	2.27	1.53	3131.1	2.00	2.02

PCR performed with a primer pair designed to amplify a fragment of the *B. xylophilus* actin gene gave a product of approximately 350bp (the expected size) from cDNA of 5 populations of *B. xylophilus* (Fig 7.2). PCR with primer pairs designed to amplify from cellulase and expansin genes produced the expected fragments of 700bp (Fig 7.3) and 470bp

(Fig 7.4) respectively from cDNA of each *B. xylophilus* population. Amplification with a primer pair targeting the pectate lyase gene generated a product of the anticipated size (700bp) from cDNA of 4 populations of *B. xylophilus* (Fig 7.5). For the BxCAN population, which failed in cDNA-PCR, PCR amplification was successful from genomic DNA. PCR using the β-1,3-endoglucanase primer pair was more problematic. Only one population (BxCAN) gave a PCR product using cDNA as a template and the size of the amplified fragment was around 750bp as expected (Fig 7.5). After testing different concentrations of $MgCl_2$ and template cDNA at different annealing temperatures a product of the anticipated size was obtained from another population (BxPOT). It was not possible to amplify any PCR product using the β-1,3-endoglucanase primer pair with gDNA as a template.

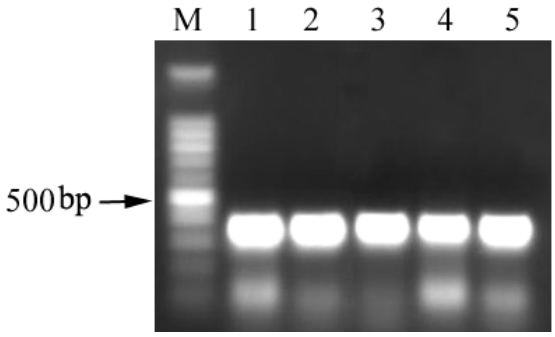

Fig 7.2 A 350bp fragment amplified from cDNA of *Bursaphelenchus xylophilus* populations with actin primer pair BxactinF and BxactinR. Lane1: BxSD; lane2: BxLYG; lane 3: BxCAN; lane4: BxJAP; lane 5: BxPOT. M: molecular size marker (100bp DNA ladder, Promega).

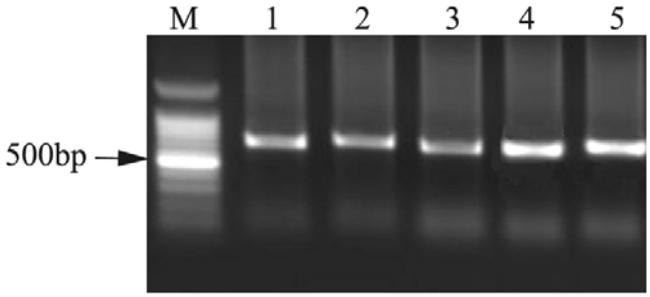

Fig 7.3 A 700bp fragment amplified from cDNA of *Bursaphelenchus xylophilus* populations with cellulase primer pair BxENG00F and BxENG00R. Lane1: BxSD; lane2: BxLYG; lane 3: BxCAN; lane4: BxJAP; lane 5: BxPOT. M: molecular size marker (100bp DNA ladder, Promega).

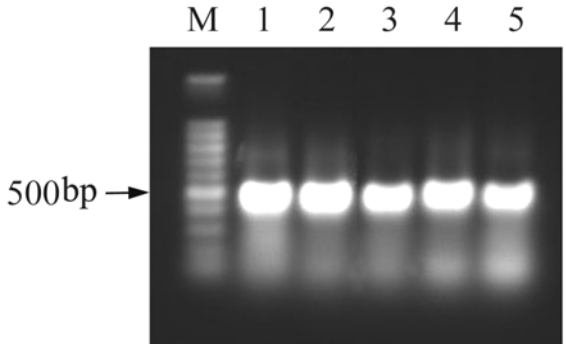

Fig 7.4 A 470bp fragment amplified from cDNA of *Bursaphelenchus xylophilus* populations with expansin primer pair BxEXPF and BxEXPR. Lane1: BxSD; lane2: BxLYG; lane 3: BxCAN; lane4: BxJAP; lane 5: BxPOT. M: molecular size marker (100bp DNA ladder, Promega).

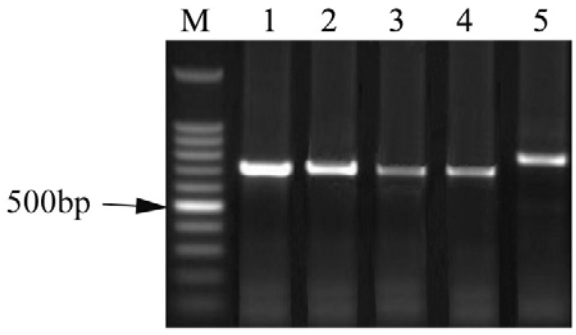

Fig 7.5 Lanes 1-4: A 700bp fragment amplified from cDNA of *Bursaphelenchus xylophilus* populations with pectate lyase primer pair BxpelF and BxpelR. Lane1: BxSD; lane2: BxLYG; lane 3: BxJAP; lane4: BxPOT. M: molecular size marker (100bp DNA ladder, Promega). Lane 5: 750bp fragment amplified from BxCAN cDNA with β-1.3-endoglucanse primer pair Bx13engF and Bx13engR.

7.3.1.2 Analysis of variation in pathogenicity related genes

The PCR products were cloned and a total of 258 white colonies were selected for analysis by colony-PCR. Plasmid DNA was extracted from at least 10 colonies showing an insert of the expected size. From these, a total of 171 inserts were sequenced. The number of the purified plasmids and sequenced plasmids for each gene are listed in Table 7.5.

DNA sequences derived from each gene were aligned using ClustalW and pairwise sequence divergences were calculated and NJ trees generated using MEGA 3.1. The pairwise sequence divergences of each gene within each nematode population are listed in Table 7.6.

Table 7.5 Number of colonies and sequences obtained from pathogenicity related genes of *Bursaphelenchus xylophilus* populations.

Gene	Number	Nematode population					
		BxSD	BxLYG	BxCAN	BxJAP	BxPOT	Total sequenced plasmids
Cellulase	Plasmid code	CS	CL	CC	CJ	CP	
	Colony for plasmid	12	12	12	12	12	34
	Plasmid sequenced	7	5	8	8	6	
β-1,3-endoglucanase	Plasmid code	ES	EL	EC	EJ	EP	
	Colony for plasmid	0	0	12	0	12	16
	Plasmid sequenced			8		8	
Petate lyase	Plasmid code	PS	PL	PC*+PCA	PJ	PP	
	Colony for plasmid	12	12	6*+10	12	12	39
	plasmid sequenced	7	8	3*+10	4	7	
Expansin	Plasmid code	ExS	ExL	ExC	ExJ	ExP	
	Colony for plasmid	12	12	12	12	12	38
	Plasmid sequenced	8	7	7	8	8	
Actin	Plasmid code	AS	AL	AC	AJ	AP	
	Colony for plasmid	10	10	10	10	10	44
	Plasmid sequenced	10	7	9	8	10	

* Gene fragment amplified from genomic DNA.

Table 7.6 Pairwise sequence divergences of pathogenicity related genes within each nematode population of *Bursaphelenchus xylophilus*.

Population	Gene				
	Cellulase	β-1,3-endoglucanase	Pectate lyase	Expansin	Actin
BxSD	0.003	NA*	0.002	0.001	0.000
BxLYG	0.009	NA	0.001	0.001	0.000
BxCAN	0.003	0.008	0.004	0.001	0.007
BxJAP	0.015	NA	0.01	0.001	0.000
BxPOT	0.006	0.001	0.002	0.001	0.003

* No sequences analysed.

The sequence divergence values for the cellulase gene within each of 5 populations ranged from 0.003 to 0.015, the pectate lyase gene from 0.001 to 0.01 and the actin gene from 0.000 to 0.007. Compared to the other populations, the sequences of cellulase and pectate lyase genes from BxJAP have relatively high divergence values of 0.015 and 0.01 respectively. The sequence divergence of the expansin gene is the same within each population. For the β-1,3-endoglucanase gene, the sequence divergence within BxCAN is greater than in BxPOT (0.008 *vs* 0.001).

A NJ tree showing the variation in 34 sequences derived from cellulase genes from five *B. xylophilus* populations is shown in Fig 7.6.

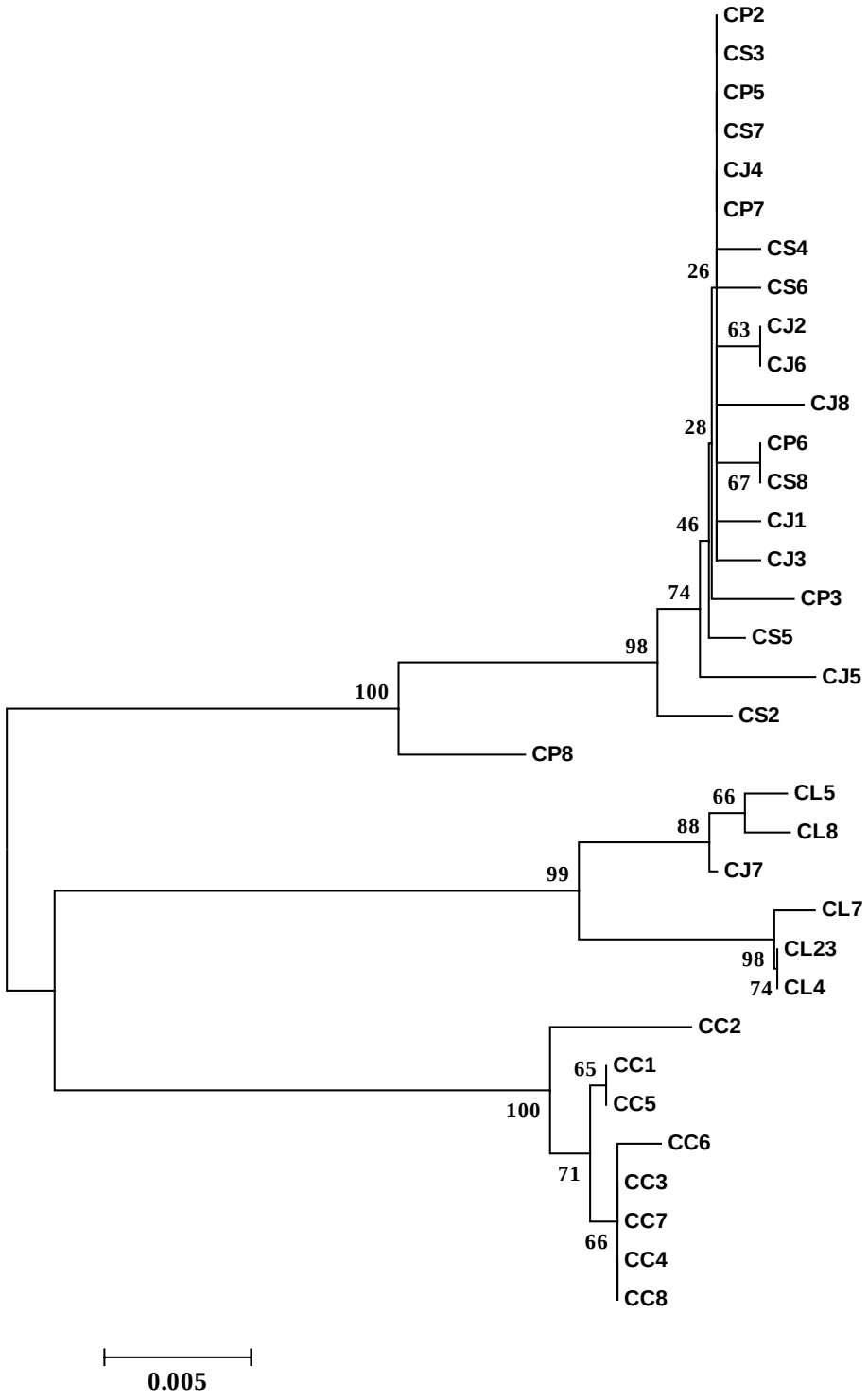

Fig 7.6 Neighbour Joining tree for 34 sequences of the cellulase gene from five *Bursaphelenchus xylophilus* populations.

The cellulase gene of *B. xylophilus* population from Canada (BxCAN) grouped with the BxLYG population from China but separated from 3 other populations (BxJAP, BxSD and BxPOT), from Japan, China and Portugal respectively, with 100% bootstrap value. The pairwise sequence divergence between the 5 populations ranged from 0.5 to 5.2%. BxLYG is different from other populations with 4.4-5.2% divergence and BxCAN different from other populations with 4.4-4.7% divergence.

The β-1,3-endoglucanase gene could only be amplified from 2 out of 5 nematode populations. A NJ tree was constructed with 16 sequences of the gene from the two populations, BxCAN and BxPOT (Fig 7.7). Eight plasmid sequences of BxCAN were split into three groups, one containing four sequences and another containing three. These two groups were clustered together but were separated from the third BxCAN group which contained just one sequence and which grouped with the sequences derived from BxPOT. The pairwise sequence divergence between these two populations was 1.5%.

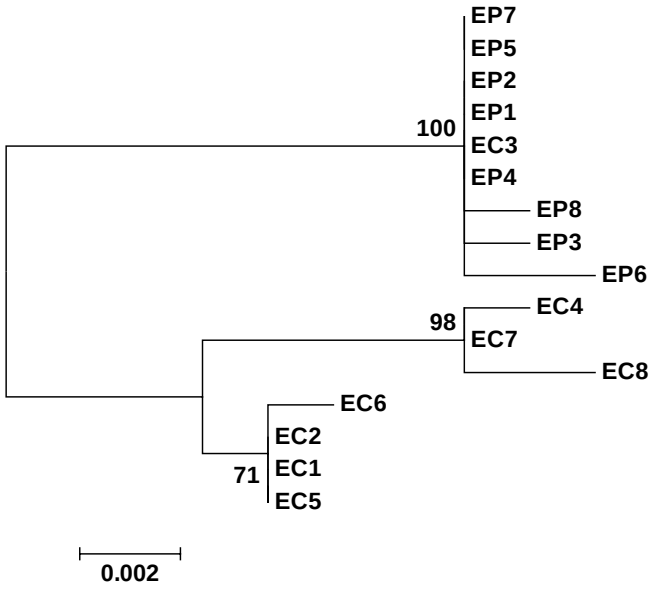

Fig 7.7 Neighbour Joining tree for 16 sequences of β-1.3-endoglucanase from two *Bursaphelenchus xylophilus* populations.

A NJ tree of 39 pectate lyase sequences from the five *B. xylophilus* populations is shown in Fig 7.8. The sequences derived from population BxJAP were separated from those derived from the other 4 populations with pairwise sequence divergence values ranging from 1.4% to 1.5%. However, only three sequences were obtained from this population which may have artificially skewed the calculations. The other four populations grouped together with very low divergence (0.2% to 0.4%). The pairwise sequence divergence between the 5

populations ranged from 0.2 to 1.5%. The pairwise sequence divergence within each population ranged from 0.1 to 1%. This implies that the sequences of the genes between and within populations are very similar to each other.

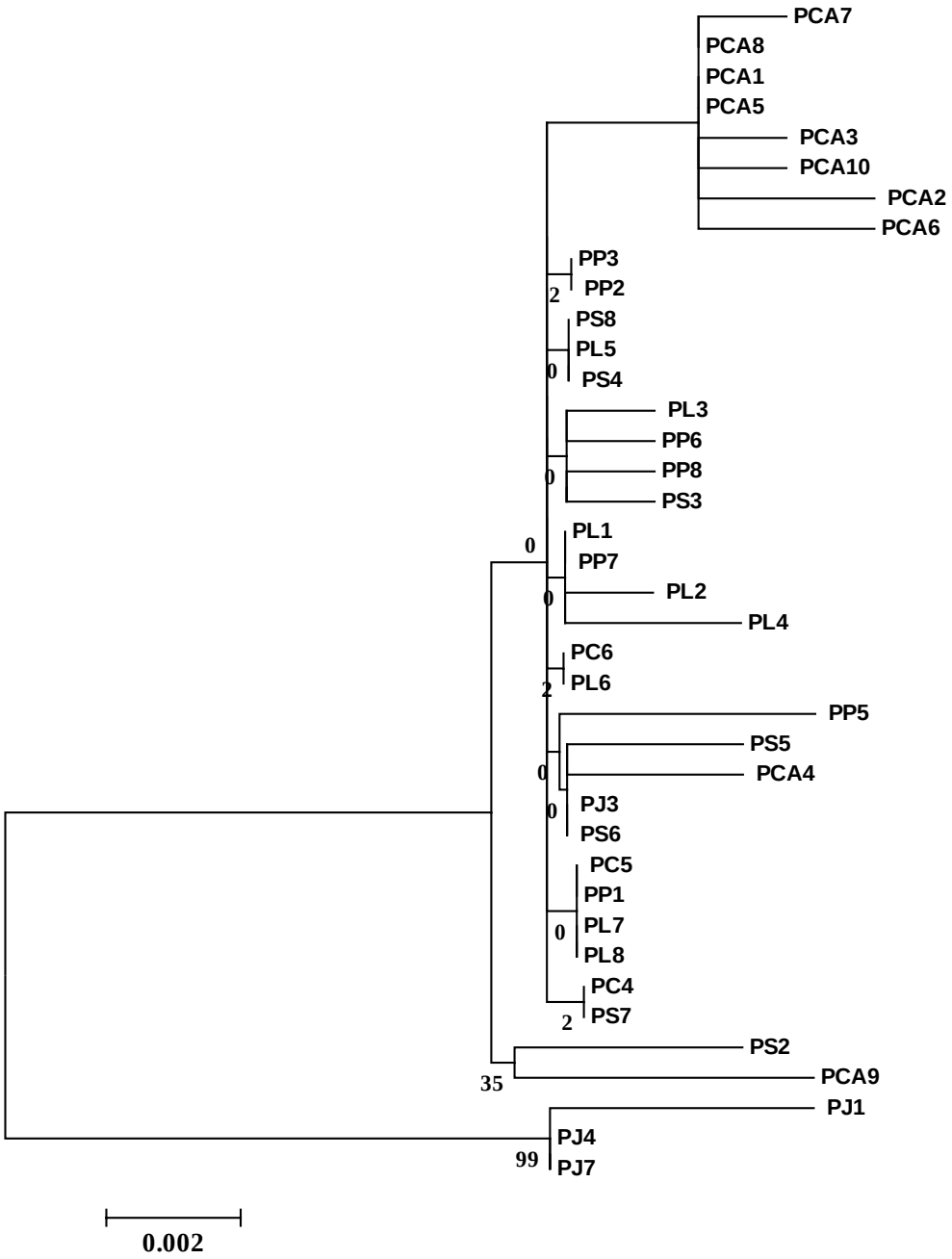

Fig 7.8 Neighbour Joining tree for 39 pectate lyase sequences from five *Bursaphelenchus xylophilus* populations.

A NJ tree of 38 sequences derived from an expansin-like gene is presented in Fig 7.9. Sequences derived from the *B. xylophilus* population from Canada (BxCAN) separated from sequences from the other 4 populations with 99% bootstrap value. The pairwise sequence divergences between BxCAN and the other 4 populations are all 4.6%. Sequence divergence

between the BxSD, BxLYG, BxJAP and BxPOT populations are all 0.1%, a reflection of the fact that all the sequences amplified from these populations are very similar. The pairwise sequence divergences within each population are also the same with the value 0.1%.

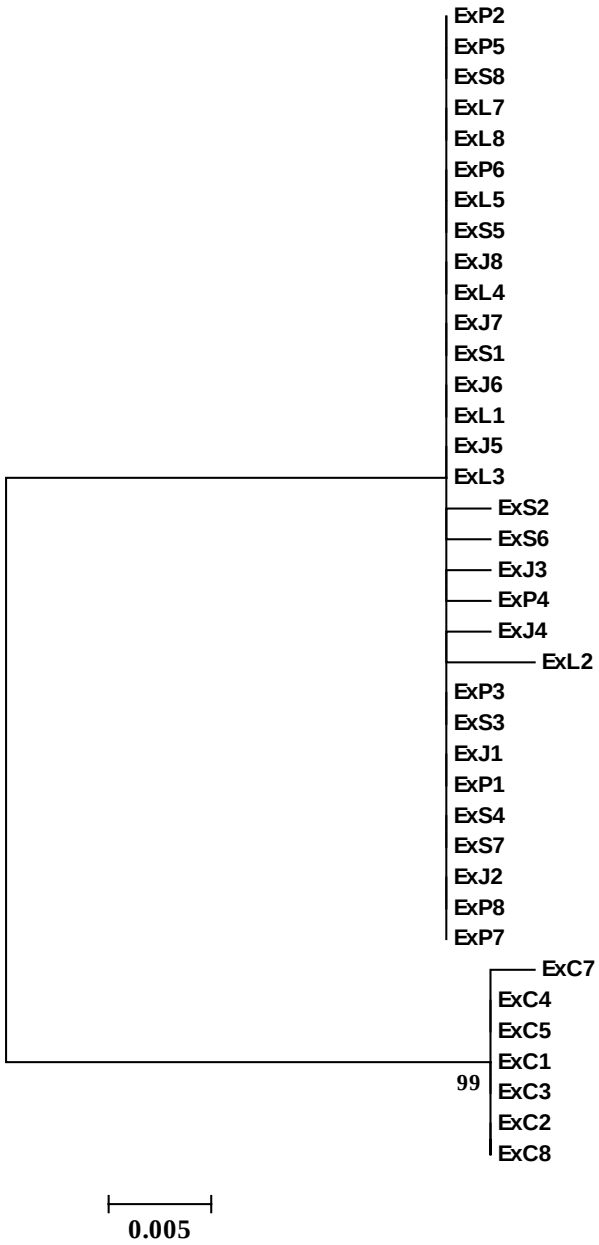

Fig 7.9 Neighbour Joining tree for 38 sequences of expansin from five *Bursaphelenchus xylophilus* populations.

For comparative purposes sequence variation in a housekeeping gene that is not secreted into the host (actin) was examined. A NJ tree of 44 sequences from actin derived from the 5 populations used in this study is shown in Fig. 7.10. No significant grouping of sequences from individual populations was observed. The pairwise sequence divergences between BxCAN and other populations ranged from 0.3 to 0.5%, BxPOT from 0 to 0.5%.

The pairwise sequence divergences within BxJAP, BxLYG and BxSD all are 0. The pairwise sequences divergences between these three populations and other populations are the same, ranged from 0 to 0.3%.

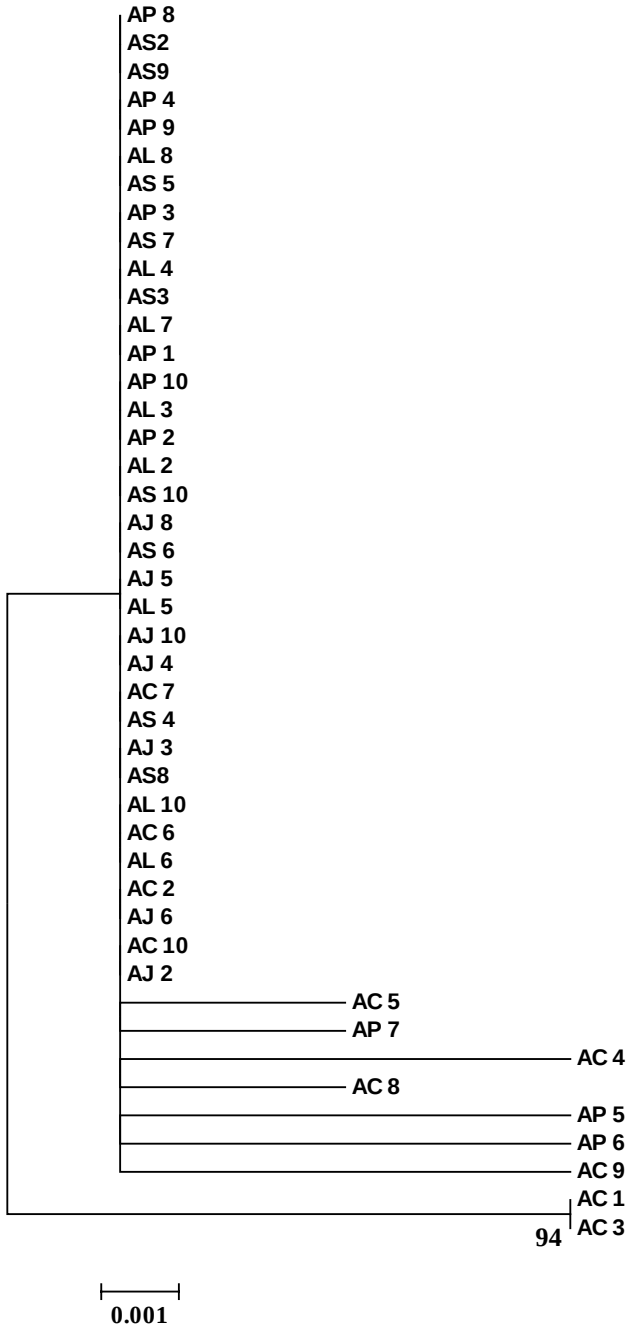

Fig 7.10 Neighbour Joining tree for actin 44 sequences from five *Bursaphelenchus xylophilus* populations.

Overall, very little sequence variation was present in any of the 4 genes tested. No clear indication as to which gene should be selected for further study from this analysis was obtained. Therefore, the expansin gene of *B. xylophilus* was selected for further functional analysis as it has not been studied previously.

7.3.2 Functional analysis of expansin-like genes of *Bursaphelenchus xylophilus* and *B. mucronatus* by *in-situ* Hybridization

7.3.2.1 Analysis of expansin-like gene from *Bursaphelenchus xylophilus*

A cDNA was identified in an EST dataset (Kikuchi *et al.*, 2007) that could encode a protein similar to expansins from the potato cyst nematode *G. rostochiensis* and other organisms. The complete nucleotide and deduced amino acid sequences of this gene, which was named *Bx-exp-1* are shown in Fig. 7.11.

Fig 7.11 Complete nucleotide and deduced amino acid sequences of *Bx-exp-1*. The 5'-and 3'-UTR are shown in lowercase, the open reading frame (ORF) is shown in capitals. The predicted signal peptide is boxed, and the gene-specific primers used for cDNA-PCR and probe synthesis (See Table 7.1) are underlined and are in bold font. The stop codon (TAA) is marked with an asterisk.

The *Bx-exp-1* cDNA is 633 nucleotides long and contains a 513-bp open reading frame (ORF). The cDNA has a 35-bp 5'-untranslated region (5'-UTR) upstream of the ATG initiation codon and a 85-bp 3'-UTR. The ORF could encode a protein of 171 amino acids with a theoretical molecular mass of 17,602 kDa and a pI of 4.58, as predicted by ProtParam (Gill & von Hippel, 1989). A short signal peptide predicted by the SignalP program (Nielsen *et al*., 1997), terminated immediately upstream of a protease cleavage site between amino acids Ala16 and Asp17.

A homology search of the amino acid sequence of BX-EXP-1 using BLASTP against non-redundant protein sequences (Altschul *et al*., 1997) showed that the protein has between 23% and 39% identity with the expansin-like proteins EXPB1 EXPB2 and EXP1 from *Globodera rostochiensis*. BX-EXP-1 was most similar (38.6% identity) to EXPB2 with lower similarity to the other two proteins (approximately 23-24% identity). BX-EXP was less similar to a putative expansin protein from *Clavibacter michiganensis* subsp. *michiganensis* with 7.0% identity, and to a cellulase celA protein which contains an expansin-like domain from *C. michiganensis* subsp. s*epedonicus* with 2.3% identity. An alignment of BX-EXP-1 with the sequences described above is shown in Fig. 7.12. Five conserved residues, marked with asterisks in Fig. 7.12, are present in all homologues from nematodes and bacteria. However, one of these residues is in the signal peptide of the *B. xylophilus* and *G. rostochiensis* sequences and will not therefore be present in the mature peptide.

A motif search (Falquet *et al*., 2002) revealed that BX-EXP-1 had two possible N-linked glycosylation sites at amino acids Asparagine (Asn)49 and Asn143, two possible Casein kinase II phosphorylation sites at amino acids Serine (Ser) 28 and Ser57, and one possible Protein kinase C phosphorylation site at Ser38. N-linked glycosylation site is also present in EXPB2 from *G. rostochiensis* and the cellulase celA protein from C. *michiganensis* subsp. s*epedonicus*. The Casein Kinase II site is also present in EXPB1, EXPB2 and EXP1 from *G. rostochiensis*. An expansin, family-45 endoglucanase-like domain is only present in EXPB1 from *G. rostochiensis* and in the putative expansin from *C. michiganensis* subsp. *michiganensis*. Whether the occurrence of these sites in these proteins is related to specific functions is unclear.

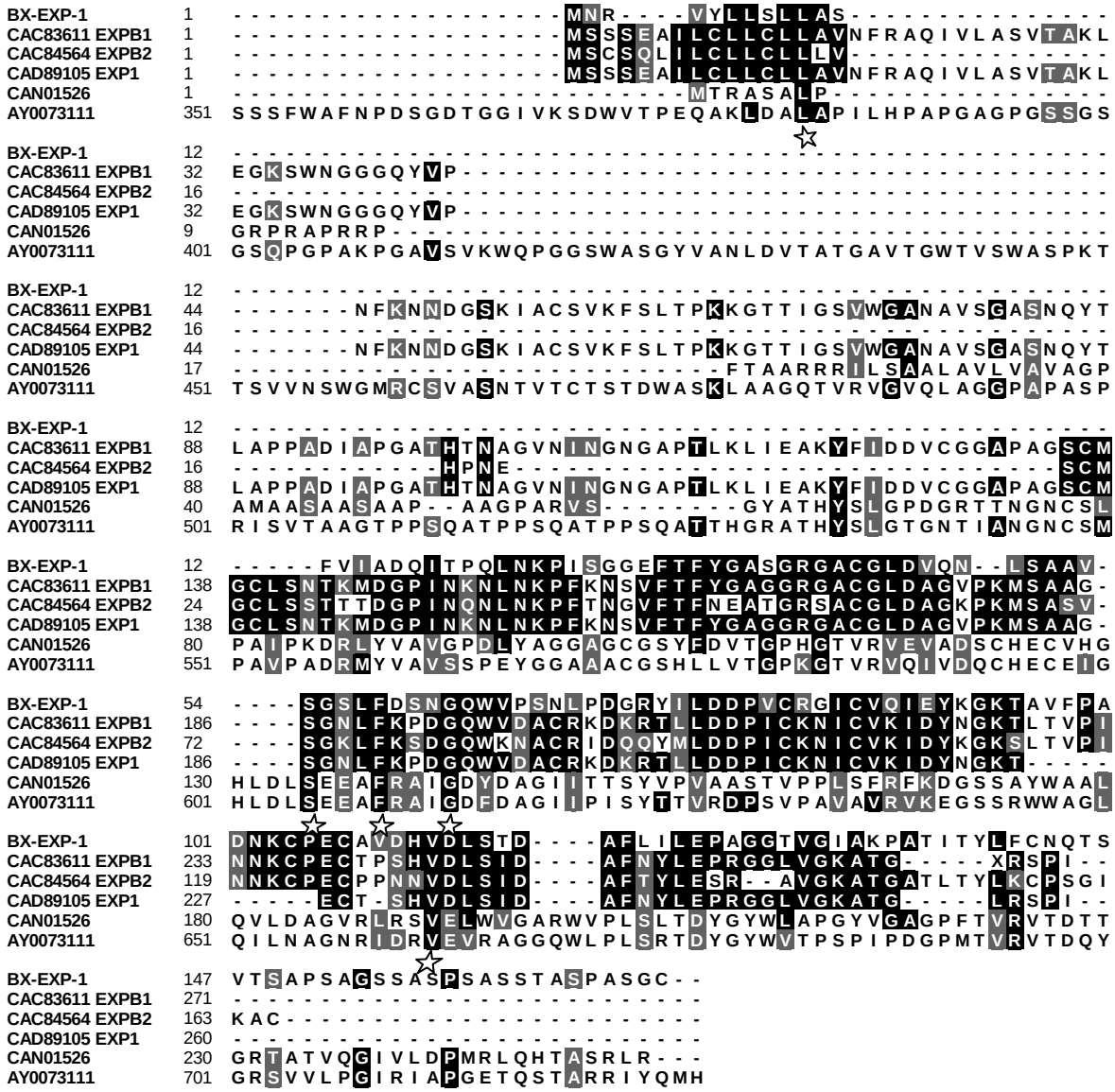

Fig 7.12 Alignment of full amino acid sequences of BX-EXP-1 with similar sequences from other nematodes and bacteria. CAC83611: *Globodera rostochiensis* EXPB1 protein; CAC84564: *G. rostochiensis* EXPB2 protein; CAD89105P: *G. rostochiensis* EXP1 protein; CAN01526: putative expansin from *Clavibacter michiganensis* subsp. *michiganensis*; AY007311: cellulase celA from *C. michiganensis* subsp. s*epedonicus.* Residues conserved in three or more sequences are outlined in black, functionally conserved residues present in three or more sequences are outlined in grey. Residues conserved in all proteins are marked with a black asterisk.

7.3.2.2 Analyzing the function of expansin-like genes in *Bursaphelenchus xylophilus* and *B. mucronatus* by *in situ* hybridization

A fragment of the expansin gene was amplified from cloned cDNA of *Bx-exp-1* using primers BxEXPF and BxEXPR1 (Figure 7.11; Table 7.1). The 250 bp fragment obtained in this reaction was visualized under UV in an agarose gel (Fig 7.13) to confirm that a fragment of the expected size had been amplified.

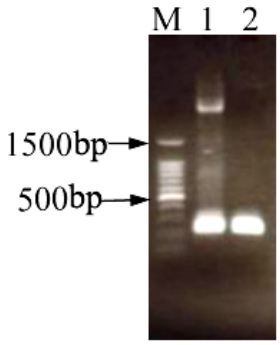

Fig 7.13 PCR products from expansin gene amplified from cDNA of *Bursaphelenchus xylophilus* (Lane 1) and *B. mucronatus* (Lane 2). M: molecular size marker (100bp DNA ladder, Promega).

Purified PCR products were used as templates in a linear PCR reaction to incorporate DIG labelled UTP. The PCR was carried out with the sense and antisense primer in separate reactions and the PCR products were checked on a 1% agarose gel to confirm that probe had been synthesized successfully (Fig.7.14). DIG labelled products show an increased molecular mass on the agarose gel (or may appear as a smear) due to the incorporated DIG.

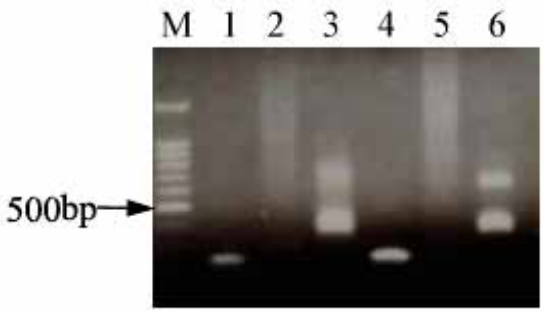

Fig 7.14 Agarose gel electrophoresis of DIG-labelled products of expansin genes amplified from cDNA of *Bursaphelenchus xylophilus* and *B. mucronatus*. Lane1-3 *B. xylophilus*; lane 4-6: *B. mucronatus*. Lanes 1 and 4 are templates; lane 2 and 5 are products from sense primers; lanes 3 and 6 are products from antisense primers. M: molecular size marker (100bp DNA ladder, Promega).

The tissue localization of the expansin transcripts was analyzed in different life stages of *B. xylophilus* and *B. mucronatus* by *in situ* hybridization. The antisense probe hybridized specifically to one of the oesophageal gland cells of *B. xylophilus* and *B. mucronatus*

(Fig.7.15). No hybridization signal was detected in the all stages of nematodes using control sense probes.

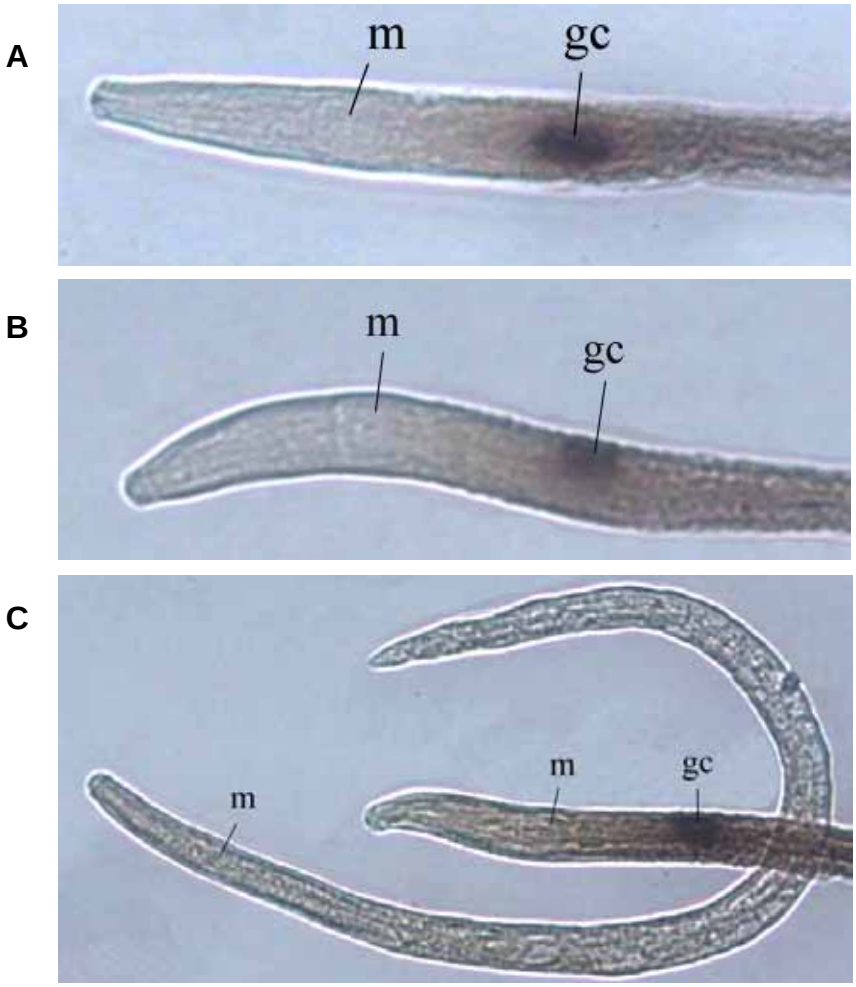

Fig 7.15 Hybridisation of the DIG-labelled antisense cDNA probe (dark staining) of expansin gene to the oesophageal gland cells of *Bursaphelenchus xylophilus* (A-B) and *B. mucronatus* (C). m: metacorpus; gc: gland cells.

7.3.3 Development of a method for RNAi in *Bursaphelenchus xylophilus*

The *in vitro* functional analyses revealed that the pathogenicity related genes were expressed in gland cells of nematodes, but for further information to be obtained the functions of the genes in nematodes need to be investigated *in vivo*. Therefore I attempted to develop a method for RNA interference (RNAi) in *B. xylophilus*. The optimum conditions for uptake dsRNA were examined and once the conditions for RNAi had been established, the technique was used to knock out selected genes and the effect on levels of transcription and on the biology of the nematode was observed.

7.3.3.1 dsRNA uptake

Nematodes reared on fungal cultures were used in these experiments. In initial experiments for examining the uptake of dsRNA by *B. xylophilus and B. mucronatus* and the effects of the neurotransmitter Octopamine on the proportion of nematodes taking up dsRNA from solution, a dsRNA synthesis reaction was carried out in which UTP was replaced with Cy3 labelled UTP to generate fluorescently labelled dsRNA. A non-endogenous gene (*gfp*) was used for these experiments. The uptake of this fluorescently labelled dsRNA was observed under light and fluorescence microscopes and the proportion of nematodes taking up dsRNA with or without Octopamine (50mM) was compared. In addition, the percentage mortality after each treatment was compared. These data are summarised in Table 7.7.

Table 7.7 Uptake of dsRNA by *Bursaphelenchus xylophilus* and *B. mucronatus.*

Treatment*	Survival of nematodes			Uptake of dsRNA		
	Alive	Dead	Percentage of live nematodes	No	Yes	Percentage of Nematodes taking up RNA
Bx -	101	11	90.2	13	40	75.5
Bx + Octopamine	105	10	91.3	45	7	13.5
Bm -	100	10	90.9	30	20	40.0
Bm + Octopamine	87	15	85.3	38	12	24.0

* Bx: *B. xylophilus*; Bm: *B. mucronatus*; - without Octopamine.

After soaking in dsRNA for 24 hr, no differences were observed in the survival of either nematode species between treatments with or without adding octopamine, but clear differences were observed in the proportion of nematodes taking up dsRNA from solution. Addition of Octopamine to soaking medium resulted in fewer nematodes taking up dsRNA from solution for both species. This is in contrast to the situation observed in cyst nematodes where Octopamine is required to induce feeding and uptake of dsRNA (Urwin *et al.*, 2002). When comparing species in the absence of Octopamine, a far greater proportion of *B. xylophilus* showed uptake of dsRNA than *B. mucronatus.*

Visualization of dsRNA taken up by the nematodes under a fluorescence microscope revealed that *B. xylophilus* took up more dsRNA than *B. mucronatus*. A strong fluorescence signal was seen in the lumen of the pharyngeal system and in the digestive system of *B. xylophilus* (Fig 7.16). The dsRNA accumulated particularly strongly in the stylet and at the end of the intestine. Weaker fluorescence signals were observed in the pharyngeal lumen and

the digestive system of *B. mucronatus* with a similar pattern of dsRNA accumulation at the end of the intestine (Fig 7.17).

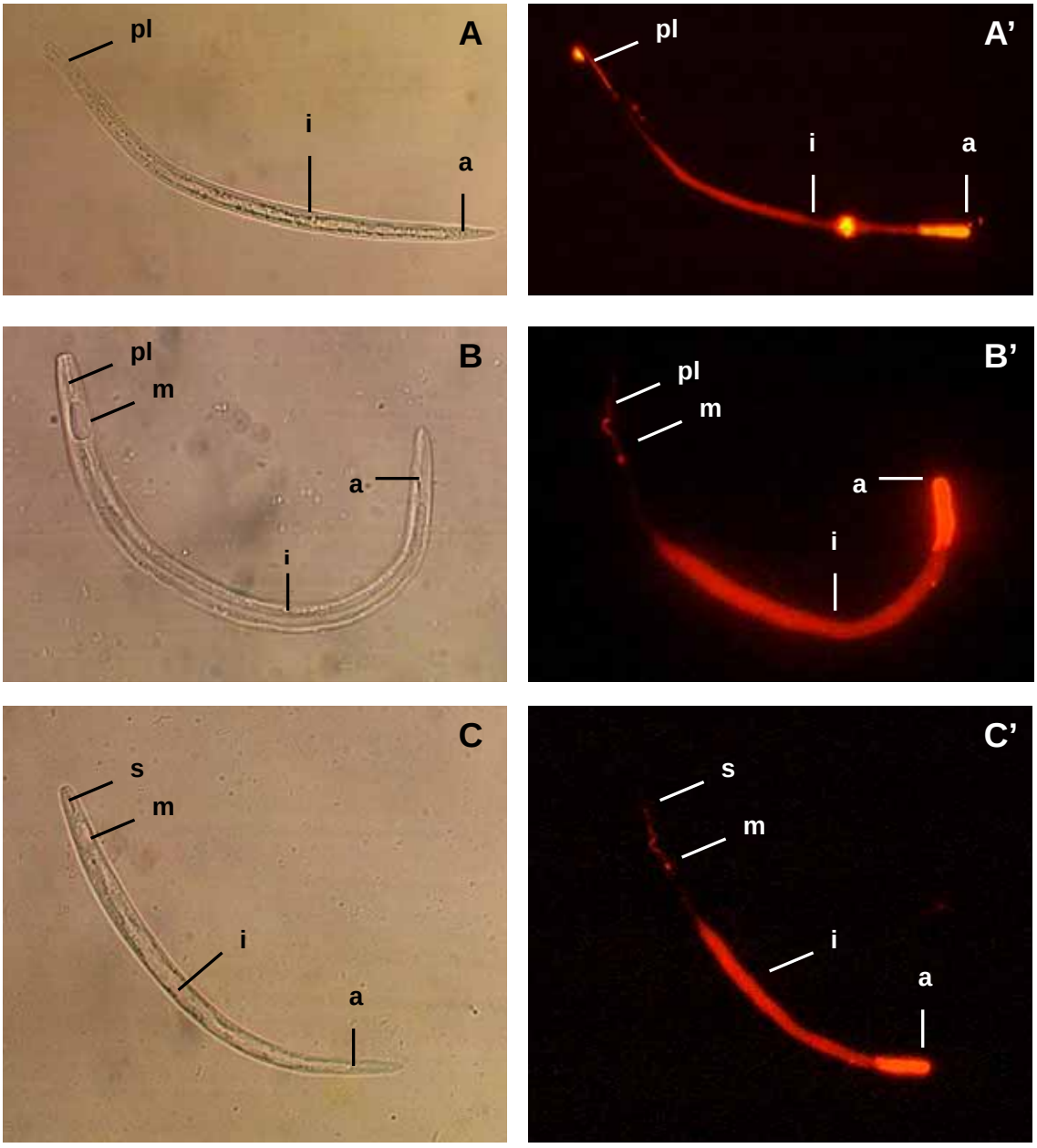

Fig 7.16 Uptake of fluorescently labelled dsRNA by *Bursaphelenchus xylophilus* after 24 hr soaking without octopamine. A, B and C, show nematodes viewed under light microscope; A′, B′ and C′ show the nematodes viewed under fluorescence microscope. Fluorescent signals appear in the lumen of the stylet, the pharyngeal lumen, the median bulb and the lumen of the intestine anterior to the anus. a = anus; i = intestine; m = median bulb; pl = pharyngeal lumen; s = stylet.

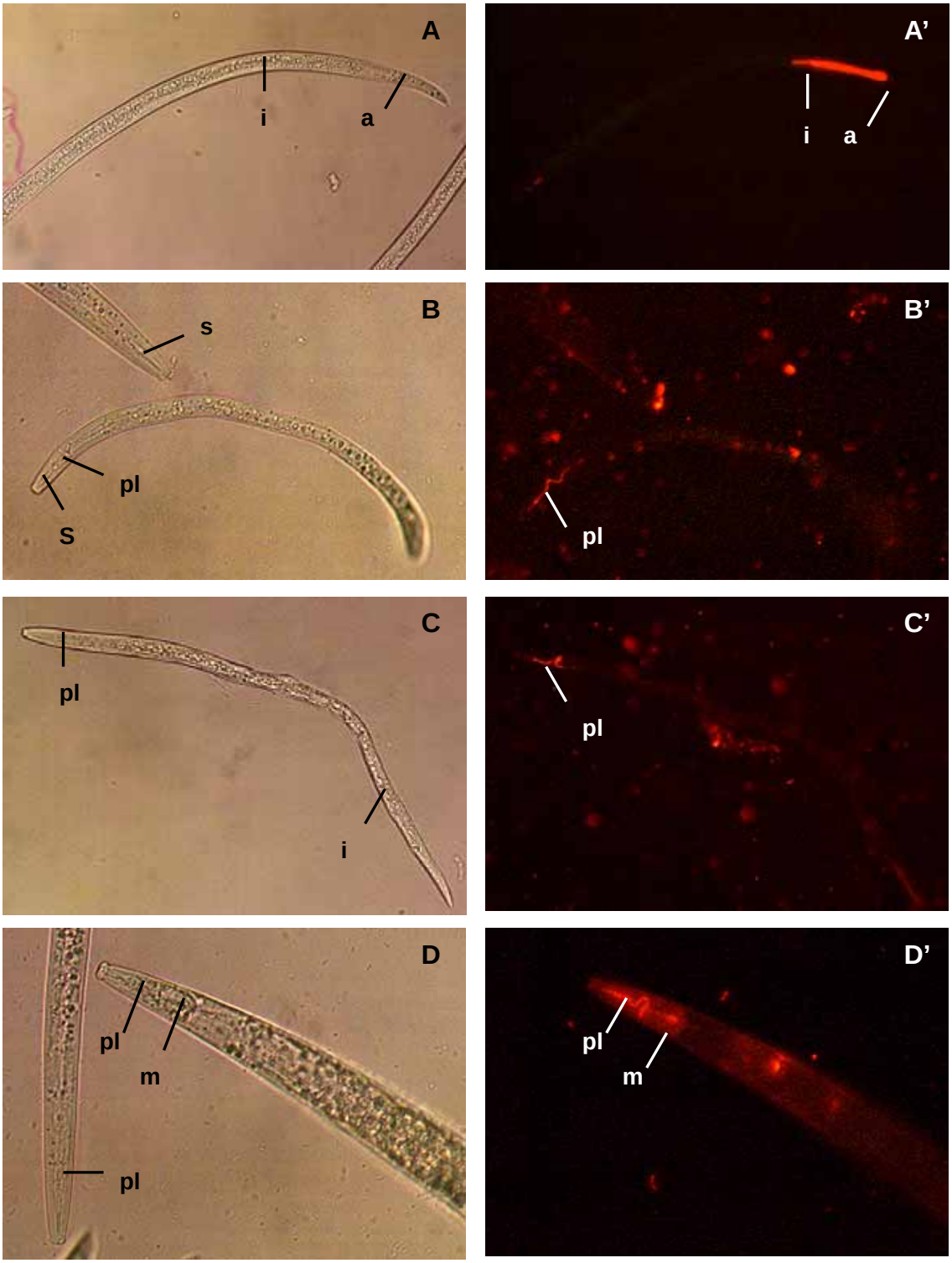

Fig 7.17 Uptake of fluorescently labelled dsRNA by *Bursaphelenchus mucronatus* after 24 hr soaking without octopamine. A, B and C, show nematodes viewed under light microscope; A′, B′ and C′ show the nematodes viewed under fluorescence microscope. Fluorescent signals appear in the lumen of the stylet, the pharyngeal lumen, the median bulb and the lumen of the intestine anterior to the anus. a = anus; i = intestine; m = median bulb; pl = pharyngeal lumen; s = stylet.

7.3.3.2 Synthesis of dsRNA of selected genes

The preliminary experiments described above showed that *B. xylophilus* takes up more dsRNA from solution after a 24 hr incubation as compared to *B. mucronatus*. I therefore chose *B. xylophilus* for functional studies using RNAi. Three genes, *β*-1,3-endoglucanse (1,3*eng)*, Ribosomal Protein Subunit 23 (*rps*23) and Green Florescent Protein (*gfp)* as a negative control were selected and primer sets were designed to allow synthesis of dsRNA. PCR reactions were carried out and the products were purified and checked on 1% agarose gel (Fig 7.18). Two PCR products were amplified from each gene with the T7 promoter sequence incorporated at the 5' end of either the sense or antisense strand.

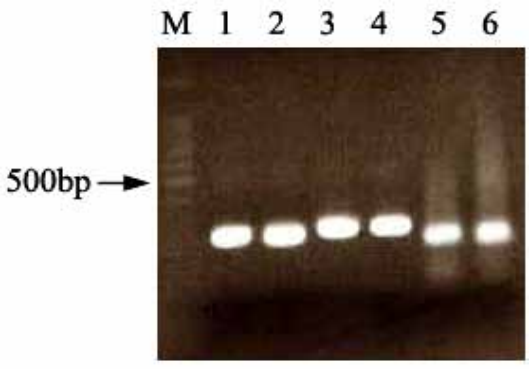

Fig 7.18 Agarose gel electrophoresis of the T7 promoter PCR products amplified from DNA templates with T7 primer pair sets. Lane 1-2: 1,3*eng* sense and antisense strand; lane 3-4: *rps*23 sense and antisense strand; lane 5-6: *gfp* sense and antisense strand. M: molecular size marker (100bp DNA ladder, Promega).

1μg of each PCR product was used in a transcription reaction and the sense and antisense RNA strands were annealed together The dsRNA were purified and quantified and checked on a 1% agarose gel (Fig 7.19).

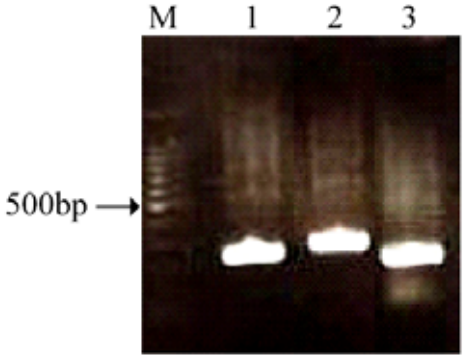

Fig 7.19 dsRNA synthesized from each gene. Lane 1: 1,3*eng*; lane 2: *rps*23; lane 3: *gfp*. M: molecular size marker (100bp DNA ladder, Promega).

7.3.3.3. RNAi

In initial experiments attempts were made to knock down expression of the *B. xylophilus rps*23 gene. Previous studies using both *C. elegans* and *H. glycines* have shown that knock out of this gene is lethal, a phenotype that should be readily scorable. In the first experiment, the freshly cultured *B. xylophilus* were collected from fungal plates and concentrated into pellets and mixed with dsRNA (*rps*23, or *gfp*, respectively) at a concentration of 1.5-2.0 μg/μl along with 0.05% gelatin and 3mM spermidine. Quantitative RT-PCR showed no difference in the levels of expression of the *rps*23 gene in nematodes exposed to dsRNA from *rps*23 or *gfp* and no difference in survival rates of the nematodes was observed for 5 days after soaking. In the later experiments, 45μl of 1mg/ml lipofectin or lipofectamine was combined with the dsRNA before soaking and added to the nematodes in an attempt to aid passage of the dsRNA across the gut wall. In these experiments an RNAi effect was observed when targeting the *rps*23 gene but not the 1,3*eng* gene.

When lipofectin was added to the dsRNA solution, the transcription levels of *rps*23 were reduced in samples exposed to *rps*23 dsRNA compared to samples exposed to *gfp* dsRNA. The PCR products obtained after 26 cycles and 30 cycles of RT-PCR are shown in Fig 7.20. While the amplification of a band from actin is similar in *rps*23 and *gfp* samples, it can be seen that levels of the *rps*23 amplicon are reduced in the nematodes exposed to *rps*23 dsRNA as compared to those exposed to *gfp* dsRNA. These data indicate a specific reduction in *rps*23 transcript in nematodes exposed to *rps*23 dsRNA.

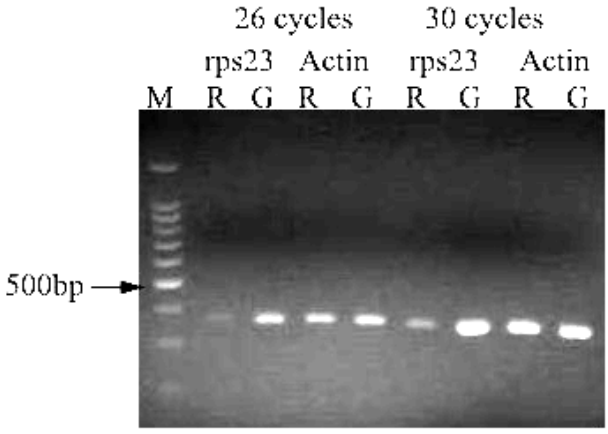

Fig 7.20 Reverse-transcription polymerase chain reaction analysis of levels of *rps*23 and *actin* in nematodes exposed to *rps*23 dsRNA as a test (R) and *gfp* dsRNA as a control (G) with lipofectin added to the soaking mixture. After 26 cycles, the *rps*23 band is present in all samples but is present at far higher levels in control samples. A band amplified from actin is similar in test and control samples. M: molecular size marker (100bp DNA ladder, Promega).

The numbers of dead nematodes resulting from incubation of dsRNA of *rps*23 and *gfp* were compared and the mortalities were calculated (Fig 7.21). As discussed above, a lethal effect of *rps*23 RNAi was expected. In this experiment, differences in the mortality rate of nematodes one day, two days and four days after soaking in *rps*23 dsRNA compared to *gfp* dsRNA solution were observed, but there was no significant difference in mortality between *rps*23 and *gfp* dsRNA treatments ($df = 1$, $F = 0.554$, $P = 0.461$).

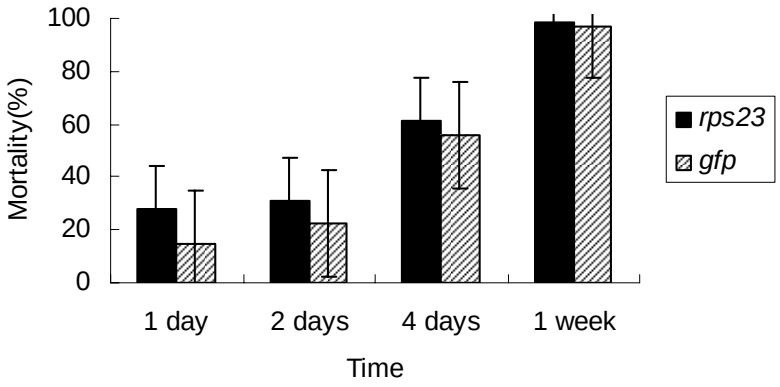

Fig 7.21 Mortality of nematodes 1 day, 2 and 4 days and 1 week after soaking in *rps*23 or *gfp* dsRNA with lipofectin.

A further experiment was performed using lipofectamine to enhance transport of dsRNA across the gut wall. Adding lipofectamine to the dsRNA solution allowed knockdown in the transcription of *rps*23 to be observed, as assessed by RT-PCR (Fig 7.22).

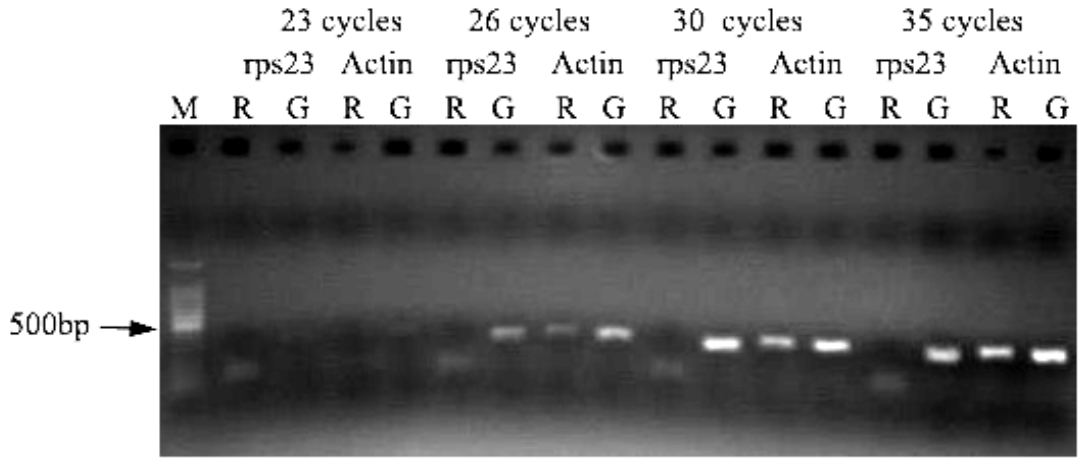

Fig 7.22 Reverse-transcription polymerase chain reaction analysis of levels of *rps*23 and *actin* in nematodes exposed to *rps*23 dsRNA as a test (R) and *gfp* dsRNA as a control (G) with lipofectamine added to the soaking mixture. After 26 cycles, the *rps*23 band is disappear in *rps*23 dsRNA treated samples but is present in *gfp* dsRNA treated samples. A band amplified from actin is similar in test and control samples. M: molecular size marker (100bp DNA ladder, Promega).

The amplification of a band from actin is similar in *rps*23 and *gfp* samples. These experiments demonstrate that RNAi effect was specific to *rps*23. The mortalities of nematodes within one week observation after recovered from *rps*23 and *gfp* dsRNA incubation are shown in Fig 7.23. The differences in the mortality rate of nematodes between *rps*23 and *gfp* dsRNA treatments were observed in 1, 2, 4 and 7 days. The mortality of *rps*23 dsRNA treatment is significantly different from that of *gfp* dsRNA treatment ($df = 1$, $F = 4.99$, $P = 0.033$).

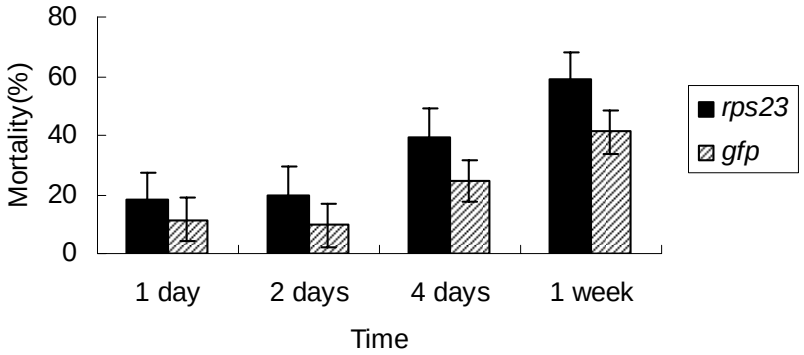

Fig 7.23 Mortality of nematodes in 1 day, 2 and 4 days and 1 week after soaking in *rps*23 or *gfp* dsRNA with lipofectamine.

In 1,3*eng* RNAi experiments, the effect of soaking in dsRNA from the target gene 1,3*eng* was compared with the effects of soaking in the non-endogenous gene (*gfp*) as a negative control. No reduction in 1,3*eng* transcription levels were observed, even using lipofectin or lipofectamine (Fig 7.24). No differences in phenotype were observed after soaking in 1,3*eng* dsRNA.

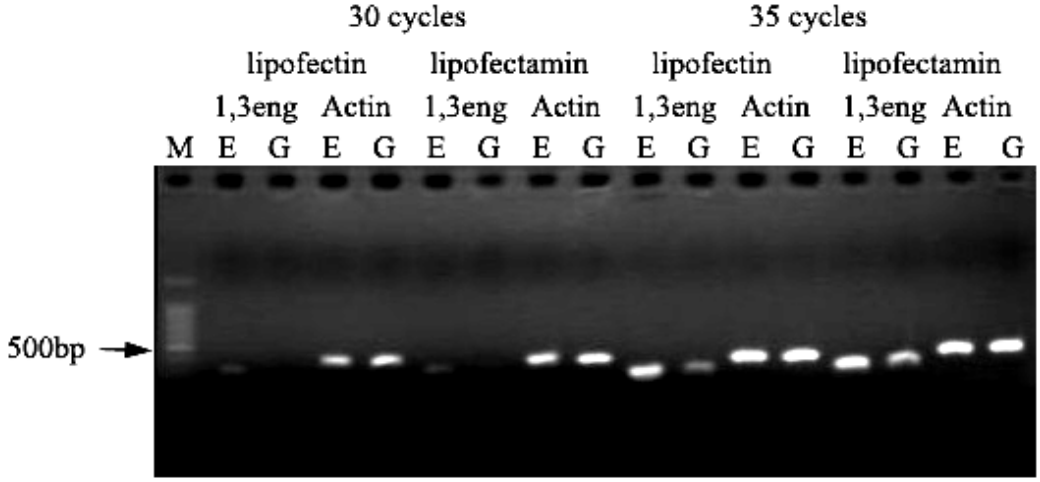

Fig 7.24 Reverse-transcription polymerase chain reaction analysis of levels of 1,3*eng* and *actin* in nematodes exposed to 1,3*eng* dsRNA as a test (E) and *gfp* dsRNA as a control (G) with lipofectin and lipofectamine. After 30 cycles, the 1,3*eng* band is present at slightly higher levels in test samples compared to control samples. A band amplified from actin is similar in test and control samples. M: molecular size marker (100bp DNA ladder, Promega).

7.4 Discussion

7.4.1 Pathogenicity related genes

Different molecular techniques have been used to identify genes that encode proteins important in host-parasite interactions (pathogenicity genes) from a range of sedentary endoparasitic nematodes. However, until recently little was known about the molecular basis of host–parasite interactions in *Bursaphelenchus* species, especially pathogenicity-associated proteins. In this work, five populations of *B. xylophilus* with a range of pathogenicity characteristics were selected and variation in four pathogenicity related genes was examined in comparison to that in a housekeeping gene (actin).

Cellulase and expansin genes were readily amplified from cDNA of each of the 5 populations. No major differences in the gene sequences between or within each of the 5 populations were observed and low divergence values were therefore obtained. However, phylogenetic analysis of the cellulase sequences showed that the BxCAN population is separated from the other 4 populations (from China, Japan and Portugal). The NJ tree derived from the expansin-like gene sequences also showed that BxCAN separated from the other 4 populations with a pairwise sequence divergence of 4.6%. These data suggest a higher separation and a higher level of divergence for the Canadian population than for the other populations.

Although β-1,3-endoglucanase genes were only amplified successfully from cDNA of BxCAN and BxPOT populations, similar findings arose from analysis of this gene. The sequence divergence within this gene was higher in the BxCAN population than in the BxPOT population.

Pectate lyase genes were amplified from cDNA of four populations of *B. xylophilus* but could only be amplified from genomic DNA of the BxCAN population. In contrast to the results for the other genes, the pectate lyase gene from BxJAP showed higher divergence compared to the other populations. The NJ tree revealed that BxJAP was separated from the other four populations which grouped together. However, very few sequences were analysed from the BxJAP population and this may have biased the calculations.

Actin genes were amplified from cDNA of all five populations and, as expected for this control gene, the pairwise sequence divergences were very low. No specific groups were revealed from a NJ tree generated from this data.

Limited information was obtained from analyzing the sequence variation in pathogenicity genes. No one pathogenicity gene showed high levels of variation that might

suggest that it is under selection pressure to diversify in order to avoid detection by the host. Although the sequences of each of the four genes between and within nematode populations are very similar to each other with low pairwise divergence values, the population from Canada (BxCAN) showed a higher variation when compared to other 4 populations from China, Japan and Portugal.

Bursaphelenchus xylophilus is considered as a native species to North America (Rutherford *et al.*, 1990) and is thought to have been introduced to Japan in the late 19th century. It subsequently spread to China, Taiwan, South Korea and, most recently, has been introduced into Portugal. In recent work tracing the origin of the Portuguese PWN isolates, the fingerprints obtained by ISSR and RAPD-PCR from 30 *B. xylophilus* isolates from the North American, East Asia and Portugal, revealed lower genetic variation within introduced populations as compared to native North American populations (Metge & Burgermeister, 2006). Although my work is more limited in scope, the findings are similar, with higher levels of variation in sequences of some of the pathogenicity genes in the North American population BxCAN compared to the other four (Asian/Portuguese) populations. In my pathogenicity tests, BxCAN showed lower pathogenicity to *P. thunbergii* seedlings with slower symptom development and lower mortality compared to the other 4 populations (chapter 6). The relationship between the variations in pathogenicity related genes of *B. xylophilus* populations and their pathogenicity to *P. thunbergii* still needs further study.

Recent RAPD-PCR analysis revealed that 24 Portuguese *B. xylophilus* isolates displayed limited genetic variation in genomic DNA, suggesting a single introduction of *B. xylophilus* into Portugal with an East Asian origin (Vieira *et al*., 2007). Low genetic distance values were also found within the subclusters of the isolates from China, South Korea and Japan (Metge & Burgermeister, 2006). Single introductions of *B. xylophilus* outside North America translocated, for example, by packaging wood are likely to have comprised a small number of nematodes which would not represent the whole genetic diversity of the species. Therefore, the native populations are likely to have a higher genetic variability than the introduced populations. In the present study, the number of populations analysed for the variation in pathogenicity related genes was very small. Future work could include a larger number of populations of *B. xylophilus.*

Limited information obtained in the research failed to give a clear signal as to which genes may be important in pathogenicity, the expansin gene of *B. xylophilus* was selected for further functional analysis as this gene had not previously been analysed in detail.

7.4.2 Functional analysis of expansin-like genes

The plant cell wall is a complex composite of cellulose microfibrils and cross-linking hemicelluloses, which are embedded in a matrix of pectic polymers and structural glycoproteins. Many organisms that use plants as a food source have to overcome the structural barrier of the plant cell wall. Various types of cell wall-degrading enzymes (CWDEs) are secreted that break covalent bonds in this polysaccharide based structure by a wide range of saprophytic and plant pathogenic bacteria, oomycetes, fungi and nematodes. Expansins, a relatively diverse protein superfamily that is widespread within the plant kingdom, directly induce extension of plant cell walls by weakening the non-covalent bonding between cellulose microfibrils and matrix glucans that help to maintain their integrity (Cosgrove, 2000). These proteins are suggested to open up the compact structure of the cell wall making it more accessible to enzymatic attack. A small number of expansin-like proteins of uncharacterised function have been found in plant-associated bacteria and fungi (Laine *et al*., 2000; Saloheimo *et al*., 2002). Recently, a gene (*Gr-Exp1*) with structural and functional similarity to plant expansins has been identified in the potato cyst nematode *Globodera rostochiensis* (Qin *et al*., 2004). This was the first non-plant gene found to have the structural and functional characteristics that define the expansin superfamily. The Gr-EXP1 protein is produced in and secreted from the subventral oesophageal gland cells of infective juveniles during invasion and migration through the root. It is thought that the expansin may assist the rapid penetration of the nematode into the root tissues.

Analysis of expressed sequence tags (EST) from *B. xylophilus* (Kikuchi *et al*., 2007) revealed a sequence encoding a protein similar to expansin-like proteins from *G. rostochiensis*. These genes have never been studied previously in *Bursaphelenchus*. The full-length cDNA of the expansin-like gene, named as *Bx-exp-1*, could encode a protein of 171 amino acids. The *B. xylophilus* expansin transcripts were specifically expressed in the oesophageal gland cells, as revealed by *in situ* hybridization. Similarity searches (BLASTP) indicated that BX-EXP-1 has between 23% and 39% identity with the expansin-like proteins Gr-EXPB1, Gr-EXPB2 and Gr-EXP1 from *G. rostochiensis*. Functional assays for expansins are extremely difficult, requiring expression of the protein in plants followed by extensometer assays (Kudla *et al*., 2005) and it was not possible to undertake these assays within the timeframe of my PhD-project.

The predicted expansin-like protein Bx-EXP-1 from *B. xylophilus* only consisted of an expansin-like domain (Kikuchi *et al.*, 2008). However, some of the expansin and

expansin-like proteins from *Meloidogyne chitwoodi* and *G. rostochiensis* have two domains, a carbohydrate binding domain (CBD) from carbohydrate binding module family II (CBM2) coupled to the expansin domain (Kudla *et al*., 2005; Roze *et al*., 2008). Genes consisting solely of the expansin domain are also present in these nematodes. CBM2 domains are also present in endogenous cellulases of *G. rostochiensis* and *Heterodera glycines* (Smant *et al*., 1998; Gao *et al*., 2004). By contrast, in spite of the availability of large EST datasets for *B. xylophilus* and *B. mucronatus* (Kikuchi *et al.,* 2007) and characterised cell wall degrading enzymes (Kikuchi *et al*., 2004; 2006) no proteins described from any *Bursaphelenchus* species contain such CBM2 domains. It is therefore possible that the CBM2 domain was acquired by an ancestor of root-knot and cyst nematodes as part of a cellulase encoding gene and that during subsequent evolution of the group it has been duplicated and transferred to the expansin-like genes. Support for this model is provided by the existence of genes consisting solely of the CBM2 domain in both cyst and root knot nematodes (Gao *et al*., 2004). The fact that the expansin-like protein from *B. xylophilus* is so similar to the expansins from *G. rostochiensis* and not like any other proteins in any other nematode species, suggests that it was acquired *via* horizontal gene transfer by a common ancestor of cyst nematodes and *Bursaphelenchus*.

The presence of expansin-like sequences in *B. xylophilus* implies that simultaneous secretion of cell-wall-degrading enzymes and expansin by a variety of plant-parasitic nematodes may increase the efficiency of plant cell wall digestion and softening by these nematodes.

7.4.3 Development of RNAi in *Bursaphelenchus xylophilus*

RNAi is being developed as a novel strategy for transgenic resistance and it has been shown that RNAi of pathogenicity genes can provide a novel basis for resistance against an endoparasitic nematode. RNAi has also been used extensively as a tool for analysis of gene function. Applying RNAi to plant-parasitic nematodes is difficult due to their small size, obligate parasitism, relatively long life cycles and because the second-stage juveniles of these parasites do not feed. However, several studies have now been carried out on these nematodes (Urwin *et al*., 2002; Bakhetia *et al*., 2005; Chen *et al*., 2005; Fanelli *et al*., 2005; Rosso *et al*., 2005; Huang *et al.*, 2006; Kimber *et al.*, 2006; Yadav *et al*., 2006; Alkharouf *et al*., 2007). No RNAi studies have previously been reported for any migratory plant-parasitic nematodes. I therefore sought to develop a method for RNAi for *B. xylophilus*.

Second-stage juveniles of sedentary endoparasitic nematodes do not feed and uptake of dsRNA by these nematodes is stimulated using the neurotransmitter octopamine (Urwin *et al*., 2002; Chen *et al.*, 2005). My tests examining the optimum conditions for uptake of dsRNA by *B. xylophilus* and *B. mucronatus* revealed that adding octopamine to soaking medium caused fewer nematodes to take up dsRNA from solution for both species. This is in contrast to the situation observed in cyst and root knot nematodes but mirrors the situation for *C. elegans,* in which octopamine inhibits the feeding process (Urwin *et al*., 2002). This demonstrates that although the structure of the nervous system may be conserved, changes in physiology may lead to considerable plasticity in function. For reasons that are still not clear, the proportion of *B. xylophilus* taking up dsRNA was far greater than that of *B. mucronatus*. RNAi studies on *B. mucronatus* may therefore require investigations into compounds that can stimulate uptake in this species.

Two genes, endo-β-1,3-glucanase and Ribosomal Protein Subunit 23 were selected for RNAi in *B. xylophilus* with the non-endogenous green fluorescent protein used as a control. The endo-β-1,3-glucanases have been characterized from *B. xylophilus* and *B. mucronatus* (Kikuchi *et al*., 2005) and may be important in the feeding process of the nematodes. Silencing of the endo-β-1,3-glucanase gene was expected to produce a reduced growth phenotype caused by a reduction in the efficiency of feeding on fungus. RNAi of the Ribosomal Protein Subunit 23 was expected to give a lethal phenotype as described for other nematodes (Alkharouf *et al*., 2007). I added 3 mM spermidine and 0.05% gelatin to the soaking solution as this may improve the efficiency of RNAi by improving stability of the dsRNA (Maeda *et al*., 2001). In addition, I added lipofectin or lipofectamine in the hope that this may improve the passage of the dsRNA across the gut wall of the nematodes. These compounds are widely used for transfection of nucleic acids to protoplasts from a range of species, including plants and oomycetes (S. Whisson, SCRI, pers. comm.) Using these conditions, an RNAi effect was observed in *B. xylophilus* when targeting the *rps23* gene. The reduction in gene transcripts revealed by RT-PCR was greater with the addition of lipofectamine compared to lipofectin. However, with both lipofectin and lipofectamine a statistically significant increase in mortality was observed but no difference was observed between the two treatments. No RNAi effect was observed in *B. xylophilus* using dsRNA derived from the endo-β-1,3-glucanase gene both in terms of phenotype and the effect on levels of the transcript.

The effectiveness of RNA interference depends on many factors, including the level and pattern of target gene expression, size and sequence composition of the dsRNA segment

and its position in the target gene (Gheysen & Vanholme, 2007). My work using fluorescently labelled dsRNA showed that dsRNA enters the nematodes but the fact that an increased effect was observed after the addition of lipofectamine or lipofectin might suggest that only limited amounts of the dsRNA pass across the gut cell membranes and enter the body of the nematodes. Further modifications of the conditions in which dsRNA is supplied to the nematodes are therefore required before RNAi can be used routinely with *B. xylophilus.*

Bursaphelenchus xylophilus has both mycetophagous and phytoparasitic phases to the life cycle. Currently no transformation system for *B. xylophilus* is available and further efforts aimed at optimizing RNAi for this species are therefore merited. Such studies could examine whether an RNAi effect is induced in nematodes feeding on transgenic fungi or plants expressing dsRNA segments. In a similar approach, genetically engineered plants expressing dsRNA targeting nematode genes have been used to silence essential genes in root-knot nematodes (Huang *et al*., 2006; Yadav *et al*., 2006). This work was aimed at generating plants resistant to root-knot nematodes. Similar approaches for pine wood nematodes are unlikely to be feasible due to the fact that the nematodes are not solely reliant on plants as a food source and because genetic engineering of *Pinus* spp. (the host plants of *B. xylophilus)* is still problematic. However, a method for RNAi in *Bursaphelenchus* would permit more detailed studies of gene function than currently possible.

Chapter 8

General discussion

The pinewood nematode *Bursaphelenchus xylophilus* vectored by *Monochamus* beetles causes pine wilt disease in China, Japan, South Korea, Taiwan and Portugal (Mamiya & Enda, 1972; Tzean & Jan, 1985b; Yang & Wang, 1989; La *et al*., 1999; Mota *et al.*, 1999) and has also been detected in pine wood in the USA, Canada and Mexico (Dropkin & Foudin, 1979; Knowles *et al.*, 1983; Dwinell, 1993). *B. xylophilus* is thought to be indigenous to North America and no significant losses are caused by nematodes in natural coniferous forests in this area, which mostly consist of resistant pine species (Robbins, 1982; Rutherford *et al.*, 1990; Bowers *et al.*, 1992; Sutherland & Peterson, 1999). However, the situation changed dramatically when *B. xylophilus* was transported to East Asian countries and devastated local forests where pine trees have had no previous exposure to the pathogen and are therefore highly susceptible to the disease.

Bursaphelenchus xylophilus is recognised worldwide as one of the most important pests in the forestry industry and is listed as a major plant quarantine objective for most countries in the world. *B. xylophilus* and *B. cocophilus* are the only two tree endoparasitic nematodes vectored by beetles (Mamiya, 1984; Gerber & Giblin-Davis, 1990). *B. xylophilus* is vectored mostly by *Monochamus alternatus* in East Asian countries while *B. cocophilus,* known as the red ring nematode, is vectored by palm weevils and devastates palm trees and coconut plantations in Caribbean islands and Central and South America (Esser & Meredith, 1987; Griffith & Koshy, 1990; Araújo *et al.*, 1998; Brammer & Crow, 2001).

B. xylophilus is characterized by phytophagous and mycophagous stages (Wingfield, 1983) and the transmission of nematodes to plants is related to maturation feeding and oviposition of beetles, which makes the life cycle of *B. xylophilus* highly complicated. Methods used to control pine wilt disease include intensified plant quarantine at international levels targeting the prevention of imported wood materials that harbour *B. xylophilus*, integrated control of vectors by chemical or biological agents, agricultural measures that include clear cutting of dead trees from infested areas and replanting with resistant *Pinus* species. The devastation of forests by *B. xylophilus* in some East Asian countries has led to millions of dollars being spent in attempts to control the spread of the disease. Money has been spent on labour to remove dead trees from (often inaccessible) mountain regions, air spraying of chemical insecticides, placing of traps baited with volatile lures to kill vector beetles and replanting pine species with limited resistance. In spite of these efforts only limited success has been achieved (Mamiya, 2004; Yang, 2004). The complexity of the life cycle of *B. xylophilus* makes the effective control of pine wilt disease particularly difficult.

Long distance transmission of *B. xylophilus* occurs as a result of human activities in which nematodes are transported to uninfected areas in dead trees or logs from infected regions. *B. xylophilus* has been detected in shipments to other regions, *e.g*. in pine wood chips imported from the United States and Canada into Finland (Rautapää, 1986), coniferous timber imported from the Asian part of Russia to Europe (Braasch *et al.*, 2001) and imported packaging wood from Japan into China (Xu *et al*., 1995). This route of introduction of *B. xylophilus* into new areas continues to be a serious threat because most shipping containers are produced from unprocessed lower quality hardwood or coniferous wood which is more likely to carry PWN or other *Bursaphelenchus* species and/or their vectors (Evans *et al*., 1996; Braasch *et al.*, 2004a). Therefore, in order to aid quarantine controls the precise and rapid identification of *Bursaphelenchus* species is important.

In an inspection carried out in Nanjing as part of plant quarantine measures, five described *Bursaphelenchus* species (*viz. B. xylophilus*, *B. mucronatus* (East Asian and European types), *B. doui, B. rainulfi* and *B. thailandae*) and one new species, *B. chengi*, were identified in imported wood packaging from eight different regions. These findings clearly confirm that *Bursaphelenchus* species are frequently found in imported packaging wood. Both the M-form (originating in Canada) and R-form of *B. xylophilus* (originating in Japan) were detected in imported packaging wood which implied that introduction of *B. xylophilus* remains a serious threat from a variety of sources. Five isolates of *B. mucronatus* (three of the European genotype and two of the East Asian genotype) were found in my inspection. This indicates that *B. mucronatus* is widespread in several continents and this species was the most frequently detected species in packaging wood (Braasch *et al.*, 2001; Tomiczek *et al.*, 2003; Gu *et al*., 2006a).

Bursaphelenchus mucronatus is considered to be non-pathogenic (McNamara & Stoen, 1988) but is suspected to cause tree death in the Far East of Russia (Kulinich *et al*., 1994). Both *B. xylophilus* and *B. mucronatus* can be transmitted by the same type of vector beetles (*Monochamus* spp.) (Mamiya & Enda, 1972; 1979). It is possible that the introduction of foreign strains of *B. mucronatus* in wood packaging could pose a risk which is not controlled by the existing quarantine measures. The morphological similarities between these two species means the identification to the species level is extremely difficult and frequently unreliable using morphological data alone (Bolla & Wood, 1999; Braasch, 2004a), particularly when samples consist solely of males or juvenile stages. Molecular methods provide useful information for species separation and are essential in order to differentiate the M-forms of *B. xylophilus* originating from North America. ITS-RFLP analysis also

allows the differentiation of the East Asian and European types of *B. mucronatus*. Species-specific PCR primer pairs have been designed to discriminate *B. xylophilus* and *B. mucronatus* (Matsunaga & Togashi, 2004). However, analysis of morphological characters remains the standard method for routine identification of the species in the genus *Bursaphelenchus* as molecular analysis also needs to refer to morphological characters. The combination of morphological and molecular observation for species description and diagnosis of species is strongly recommended for *Bursaphelenchus* species.

A number of phytosanitary regulations have been implemented by EU countries aiming at the prevention of the introduction of *B. xylophilus* and its vectors from infected countries into non-infected countries through imported wood products during international trade. However, *B. xylophilus* was discovered on *P. pinaster* in Portugal (Mota *et al.*, 1999) and it has been shown that this originated from East Asia (Metge & Burgermeister, 2006; Vieira *et al*., 2007). The failure to prevent the accidental introduction of *B. xylophilus* into the EU demonstrated that more intensive plant quarantine measures are needed, particularly in terms of border or post-border inspections for packaging wood. After the initial detection of *B. xylophilus* in Portugal in 1998, a quarantine area and a safety buffer area were established. However, the latest survey revealed a significant increase in the number of declining trees within the infected zone. New prevention measures were therefore implemented by the EU including cutting and removal of all the pine trees from a 3 km phytosanitary strip surrounding the quarantine area (Rodrigues, 2006). The permanent establishment of PWN in Portugal could become a serious problem for the European timber industry if *B. xylophilus* can not be eradicated or its spread restricted (Braasch, 2000b). Therefore, more strict quarantine regulations are needed to prevent the importation of *B. xylophilus* and other non-indigenous *Bursaphelenchus* species to non-infected countries or regions through international trade.

Plant quarantine is considered as the most effective measure to prevent the long distance transmission of *B. xylophilus* (Braasch *et al.*, 2004) and various regulations are legally implemented by different countries in order to protect their local forestry. Although the EU has restricted the import of pine wood products from North America, Japan, Korea and China and other regions of Asia, the introduction of *B. xylophilus* from East Asia to Europe shows that plant quarantine alone can not be relied on for preventing the transmission of *B. xylophilus*. Once *B. xylophilus* been introduced into a previously non-infected region, other strategies are required in order to eradicate pine wilt disease, restrict its spread and thereby decreasing forest damage.

Bursaphelenchus xylophilus can be transmitted over relatively short distances by *Monochamus* spp. in natural conditions. The nematodes are transferred as fourth-stage dispersal juveniles which congregate throughout the tracheal system of beetles (Enda, 1994; Aikawa & Togashi, 2000). The invasion of *B. xylophilus* into new host plants occurs during maturation feeding of beetles (Mamiya & Enda, 1972; Linit, 1988; Edwards & Linit, 1992) and vector control is therefore considered as an important control strategy for pine wilt disease.

The epidemiology of pine wilt disease is directly related to the initial number of nematodes being carried after beetle emergence and is also related to various physical, chemical and environmental factors. Jikumaru and Togashi (2001) showed that the initial nematode load, the nematode departure efficiency and the nematode transmission efficiency had significantly positive influences on the number of nematodes transmitted into pine twigs. *M. alternatus* was the major vector of *B. xylophilus* in several East Asian countries (Japan, China, South Korea and Taiwan). *M. galloprovincialis* was confirmed to transmit *B. xylophilus* in Portugal (Sousa *et al*., 2001). *Monochamus carolinensis*, *M. mutator*, *M. scutellatus* and *M. titillator* are vectors for *B. xylophilus* in North America (Dropkin *et al.*, 1981; Linit 1988; Edwards & Linit, 1992). *M. alternatus* was the most efficient vector of *B. xylophilus* when compared to the North American beetles (Linit *et al*., 1983). Although other insects from genera in Cerambycidae and Coleoptera were also found to carry *B. xylophilus* and many of the *Monochamus* spp. from conifers was reported as being associated with non-pathogenic *Bursaphelenchus* spp., the interactions between these insect vectors and nematodes in natural conditions are still unclear.

My two year observations of the dynamics of *M. alternatus* emergence, nematode carrying and nematode transmission by beetles suggested that air spraying of chemicals or use of allures targeting beetles should start earlier in the year and continue for a long time because beetles that emerge early in the season are very likely to carry nematodes and because beetles can transmit nematodes to pine trees throughout their life. The chemical control strategy used for insect vectors would be less effective without precise knowledge of the dynamics of beetle emergence in an infested region.

Theoretically, the most effective method for PWN control is replanting with resistant *Pinus* species or other coniferous species. The resistance screening systems depend on the aggressiveness of the nematode populations and their pathogenicity to plants (Ikeda, 1984). Investigations on pathogenicity of *B. xylophilus* to *Pinus* species have been carried out artificially under both field and laboratory conditions and often yielded very variable results,

especially in the seedling tests (McNamara, 2004). Pathogenicity studies with *Bursaphelenchus* species are extremely difficult to perform accurately due to the difficulties of simulating natural inoculation of beetle vectors. Thus, different inoculation tests were designed during this project in order to evaluate the effects of various factors on the pathogenicity of *B. xylophilus* to *P. thunbergii,* and to evaluate the pathogenicity of different *B. xylophilus* populations to *Pinus* spp. These included simulating natural nematode infection through beetle vectors and artificial inoculation of dispersal juveniles collected from beetles. From the results obtained, I conclude that replacing artificial inoculation with natural inoculation in resistance screening is not an immediate option because the number of nematodes transmitted to the plants during beetle feeding was impossible to control precisely.

My inoculation tests with varying nematode numbers showed that the number of nematodes inoculated to plants had important effects on the pathogenicity of *B. xylophilus* to *P. thunbergii.* The artificial inoculation method was considered to be a practical way to investigate the pathogenicity of *B. xylophilus* on *Pinus* species but many factors need to be considered. Artificial inoculations of *B. xylophilus* should use quantities of infective nematodes similar to the number of nematodes delivered to a plant by beetles in nature. My four years of experiments suggest that 200 nematodes per plant is the appropriate number. This number is closer to the maximum number (350) of nematodes transmitted by one beetle on one day under natural conditions (Li *et al.*, 2007). The results obtained using inoculum of 200 nematodes per plant in pathogenicity tests by artificial inoculation may be more convincing when compared to those that use inoculation with thousands of nematodes.

The pathogenicity tests with nematodes isolated from beetles or wood logs, cultured on fungus for one generation or multiple generations, and exposed to different treatments prior to inoculation, showed that the plants inoculated with non-surface sterilised nematodes that originated from beetles or nematodes cultured for one cycle on fungus showed faster symptom development and higher mortality rates compared to those inoculated with surface sterilized nematodes from beetles or wood logs and cultured for one generation or multiple generations on fungus. This suggested that the presence of other microorganisms on the nematode surface or chemical cues from beetles may have an effect on the sensitivity of plants to nematode infection. Further research could focus on the studies of chemical compounds which control the exodus of nematodes from beetles and the subsequent invasion into the tree. Such studies may provide new strategies for disease control.

I analysed resistance of various tree species against *B. xylophilus* over two years using artificial inoculations of 200 fungus-cultured nematodes that were not surface sterilized. Five populations of *B. xylophilus* were used in these studies on *P. thunbergii* and three other *Pinus* species. These studies showed that *P. taeda* and *P. elliotti* showed highest resistance/tolerance against *B. xylophilus* and that these could be used in replanting programmes in China to protect against pine wilt disease. *Pinus massoniana* was less susceptible to *B. xylophilus* infection compared to *P. thunbergii* which is considered the most susceptible *Pinus* species to *B. xylophilus*. The susceptibility of *P. massoniana* to *B. xylophilus* has also been demonstrated in previous studies in China (Bai & Cheng, 1993; Liu & Feng, 1994; Xu *et al*., 1996; Wang *et al.*, 1997). The vast forest area of *P. massoniana* in southeast China therefore face a serious threat from *B. xylophilus* (Yang, 2004).

Although the damage caused by *B. xylophilus* is known throughout the world (Bergdahl, 1999), little is known about the molecular mechanisms underlying nematode pathogenicity and host–parasite interactions. Identifying candidate genes related to pathogenicity of the nematode will provide breeding strategies to develop pine varieties with broad and durable resistance, and thus contribute greatly to the efficient management of the pine wilt disease. A series of candidate pathogenicity genes have been identified in recent studies on *B. xylophilus* Expressed Sequence Tags (Kikuchi *et al*., 2007). However, although variations in the sequences of pathogenicity genes may be important in pathogenicity characteristics of pathogens, nothing was known about variation in the candidate pathogenicity genes in *B. xylophilus.* Therefore I examined variation in pathogenicity genes of five populations of *B. xylophilus*, which demonstrated a range of pathogenicity characteristics. The variation in four pathogenicity related genes (β-1,3-endoglucanase, cellulase, expansin and pectate lyase) was examined for these five *B. xylophilus* populations. However, no one pathogenicity gene showed high levels of variation in the nematode populations which might suggest that it is under selection pressure to diversify in order to avoid detection by the host.

Although the sequences of each of the four genes between and within nematode populations are very similar to each other with low pairwise divergence values, the population from Canada (BxCAN) showed a higher variation when compared to other 4 populations from China, Japan and Portugal. The pathogenicity tests of BxCAN on *P. thunbergii* seedlings showed slower symptom development and lower mortality compared to that of the other four populations. The North American native populations are likely to have a higher genetic variability than the introduced populations. The relationship between the

variations in pathogenicity related genes of *B. xylophilus* populations and their pathogenicity to *P. thunbergii* still needs further study.

My efforts in trying to find a clear signal as to which genes may be important in pathogenicity of *B. xylophilus* failed due to the limited information obtained in analysing four pathogenicity related genes. Therefore I focused further efforts on an expansin-like gene of *B. xylophilus* which had not previously been analysed in detail. The expansin transcripts of *B. xylophilus* were specifically expressed in the oesophageal gland cells as revealed by *in situ* hybridization. The predicted expansin-like protein Bx-EXP-1 from *B. xylophilus* only consisted of an expansin-like domain (Kikuchi *et al.*, 2008) and may assist the rapid penetration of the nematode though plant cells. Functional analysis of pathogenicity related genes will provide knowledge for better understanding of the interactions between host and parasites and may provide new strategies for disease control.

RNA interference (RNAi) has been developed for use as a tool for analysis of gene function. RNAi was also developed as a novel strategy for transgenic resistance and it has been shown that RNAi of pathogenicity genes can provide a novel basis for resistance against an endoparasitic nematode (Bakhetia *et al*., 2005; Huang *et al*., 2006). Resistance is achieved by a plant delivering a dsRNA that targets a nematode gene and induces a lethal or highly damaging RNAi effect on the parasites. The effectiveness of RNA interference depends on many factors, including the level and pattern of target gene expression, size and sequence composition of the dsRNA segment and its position in the target gene (Gheysen & Vanholme, 2007). No RNAi studies have previously been reported for any migratory plant-parasitic nematodes; therefore I tried to develop a method for RNAi for *B. xylophilus*.

Analysis of the uptake of dsRNA showed that octopamine inhibited uptake of dsRNA by *B. xylophilus*. This is in direct contrast to the situation for sedentary endoparasitic nematodes where octopamine stimulates uptake of dsRNA from solution (Chen *et al*., 2005). The proportion of *B. xylophilus* taking up dsRNA was also far greater than that of *B. mucronatus.* The reasons for this still need to be clarified in further research. Two genes, endo-β-1,3-glucanase and Ribosomal Protein Subunit 23 (*rps*23) were selected for RNAi because silencing these two genes in *B. xylophilus* may produce a readily scorable phenotype. I anticipated that silencing endo-β-1,3-glucanase would lead to a reduction in the efficiency of feeding on fungus while silencing *rps*23 should give a lethal phenotype. I obtained limited success in silencing the genes when 3 mM spermidine, 0.05% gelatin and lipofectin or lipofectamine were added into the dsRNA-nematode mixture. A phenotype as a result of the RNAi effect was observed in *B. xylophilus* when targeting the *rps*23 gene but not in the

β-1,3-endoglucanase gene. Further modifications of the conditions in which dsRNA is supplied to the nematodes are required before RNAi can be used routinely with *B. xylophilus.*

Bursaphelenchus xylophilus is characterised by phytophagous and mycophagous phases and a transformation system is currently unavailable for this nematode. Further efforts aimed at optimizing RNAi for *B. xylophilus* could examine whether an RNAi effect is induced in nematodes feeding on transgenic fungi producing dsRNA targeting a nematode gene. It is possible that a combination of developing a novel strategy for transgenic resistance of pines, possibly using RNAi, with the existing control measures might provide a more effective and durable basis for further control of *B. xylophilus.*

The North American indigenous *B. xylophilus* was transported to Japan by means of contaminated wood products (Yano, 1913; Nickle *et al*., 1981; Mamiya, 1983; Malek & Appleby, 1984) and has caused damage to Japanese forestry and billons of dollars has been spent. The discovery of PWN in Nanjing which is the capital of east province Jiangsu in China (Cheng *et al*., 1983), originally led to serious attempts from the government to control the disease. However, the complicated life cycle of *B. xylophilus* made control extremely difficult, particularly where financial support was limited. Failures in strategies used to control pine wilt disease are demonstrated by the further spread of the disease to 6 provinces (Yang, 2004), and this spread is continuing from eastern provinces to south and southwestern provinces of China. Millions of trees have been killed by *B. xylophilus* but no effective control measures are available once the disease has appeared.

From my 4 years research, I think plant quarantine is an important measure that can help to prevent the importation of *B. xylophilus* to uninfected regions. The rapid and precise identification of *Bursaphelenchus* species needs the combination of experienced taxonomists with the molecular technicians. PCR-based molecular identification is suggested to be a useful tool to confirm the morphological identification. Once the *B. xylophilus* introduced into one region, clear cutting of dead or dying pine wilt tree in the field in the disease year is an important measure that may help to prevent the spread of disease by beetles. Any un-cut wilted trees left in the mountain regions and untreated dead wood can form a reservoir of disease for the following year as beetles will emerge carrying large numbers of nematodes that they will deposit when they start maturation feeding on new young twigs. Although it is labour-intensive and costly, the disease is not prevented if no money is available to cut the dead trees in the mountain regions.

Other alternative measures for control pine wilt disease are also suggested including biological control of nematodes and vectors (Shimazu *et al*., 1995; Xu *et al.*, 2002; Lai *et al*., 2002; Shimazu, 2004), use of insect attractants (Jiang *et al*., 1998; Zhao *et al*., 2000a) and plant resistant *Pinus* species. However, the efficiency of biological control is currently low and can not prevent the disease spreading. Breeding of resistant *Pinus* species is currently difficult. Few successes have been achieved using traditional breeding systems because the pathogenicity tests for evaluating the virulence of *B. xylophilus* populations produced variable results (McNamara, 2004). The information obtained from my pathogenicity tests may give some useful advice for resistance screening of *Pinus* species.

Summary

The pinewood nematode *Bursaphelenchus xylophilus* vectored by *Monochamus* beetles causes pine wilt disease in China, Japan, South Korea, Taiwan and Portugal and has also been detected in pine wood in the USA, Canada and Mexico. *B. xylophilus* is native to North America where natural coniferous species are resistant to nematodes. The control strategies for pine wilt disease include intensified plant quarantine, integrated control of vectors, agricultural measures and replanting with resistant *Pinus* species.

Bursaphelenchus xylophilus can be spread over long distances *via* the wood used for the packing of shipped commodities. Plant quarantine is an important measure to prevent the introduction of *B. xylophilus* from infected regions into uninfected regions through imported wood material that harbours nematodes and/or vector insects. Five described *Bursaphelenchus* species and one undescribed species were identified from 13 imported wood packaging samples originating from eight countries and regions during my participation in a quarantine inspection in Nanjing during 2003 and 2004. The described *Bursaphelenchus* species were identified by their morphology, morphometrics data and ITS-RFLP patterns. Two samples were identified as *B. xylophilus*; one from Canada (the M-form) and another from Japan (the R-form). Five samples were identified as *B. mucronatus*; three belonged to the European genotype and two to the East-Asian genotype. Two samples (from Germany and Korea) were recognized as *B. rainulfi*; two samples (from Korea and Japan) were identified as *B. doui* and one sample from Hongkong was identified as *B. thailandae.* Extra morphometric data were added for the identified species, especially with respect to the spicule morphometrics. The CDA of morphometrical data enabled discrimination of the five described species by nine male and eight female characters. The characters used for this analysis corresponded partly with the main taxonomic-informative characters for the genus *Bursaphelenchus*, *viz.* body length, stylet length, spicule length, a, c and c' values. The phylogenetic trees constructed from the D2D3 sequence data supported the division of East Asian and European genotype groups of *B. mucronatus*. *Bursaphelenchus doui* grouped with *B. xylophilus* and *B. mucronatus*, all members of the xylophilus group, which was separated from *B. rainulfi* and *B. thailandae.*

The new described species, *Bursaphelenchus chengi*, is characterised by the medium body size in both sexes, the presence of only two incisures in the lateral field and the robust

and strongly curved spicules with broad and blunt cucullus. The spicule lamina is angular distally, the rostrum digitate and the condylus rounded. The tail is arcuate with a pointed terminus. The bursa is usually truncate with the posterior margin indented in some specimens or rounded with a fine axial point. Females have a small vulval flap formed by a short extension of the cuticle of the anterior lip, and a conical tail that gradually tapers to an almost straight or slightly recurved, pointed or rounded terminus. Because of the presence of two lateral lines, similar spicule shape, tapering female tail and the presence of a small vulval flap, *B. chengi* is grouped in the *abietinus*-group *sensu* Braasch, together with *B. abietinus*, *B. antoniae*, *B. hellenicus*, *B. hylobianum* and *B. rainulfi*. ITS-RFLP profiles support the new species, and phylogenetic analysis of the 28S rDNA D2/D3 domain sequence places it close to *B. antoniae* and other species of the *abietinus*-group.

Bursaphelenchus xylophilus can be transmitted to host plant by beetles through maturation feeding or oviposition. The emergence of the vector *Monochamus alternatus* and the transmission of *B. xylophilus* through vector feeding on pine twigs were monitored during 2004 and 2005 in Nanjing. The emergence started from late April to the end of June and peaked from late May to early June. There were 438 and 927 adults collected in 2004 and 2005, respectively, and approximately 70% of the beetles emerged during the peak period. Visual estimation of the nematode burden on vectors by observation of the atrium of the first abdominal spiracle gave unreliable information. The percentage of beetles carrying PWN of the total number of emerged beetles was between 20 and 30%. Seventy percent of the nematodes were released from beetle cadavers after three days of extraction. The sex and longevity of the beetle had no significant relationship with the number of nematodes that remained in cadavers. Transmission of nematodes into pine twigs through beetle feeding started 10 days after emergence of the vector. The period of nematode transmission could last for up to 79 days after beetle emergence. Two types of nematode-transmission curve were found by measuring the number of nematodes transmitted into pine twigs per day. The unimodal pattern peaked between 3 and 6 weeks after adult emergence; the bimodal pattern had two transmission peaks: one between 2 and 3 weeks, and another between 5 to 7 weeks after beetle emergence.

The most effective control for *B. xylophilus* is replanting with resistant *Pinus* species or other coniferous species. The resistance screening systems depend on the aggressiveness of the nematode populations used and their pathogenicity to plants. The variations in results obtained from previous pathogenicity tests were criticized. Therefore, different pathogenicity experiments were designed to evaluate the factors that might affect the pathogenicity of *B.*

xylophilus to *P. thunbergii*, and to evaluate the pathogenicity of different *B. xylophilus* populations to *Pinus* spp. These included simulating natural nematode infection through beetle vectors and artificial inoculation of dispersal juveniles collected from beetles.

The variable results obtained in two years of experiments showed the impracticality of using nematode-carrying beetles, simulating the natural transmission, as an inoculation tool in pathogenicity tests. Artificial inoculation is the only practical way to investigate the pathogenicity of *B. xylophilus* on *Pinus* species but many extra factors need to be considered. The inoculation density has important effects on the expression of the pathogenicity of *B. xylophilus* to *P. thunbergii.* In artificial inoculations 200 nematodes per plant was found to be the appropriate density, which is close to the maximum number (350) of nematodes transmitted by one beetle on one day under natural conditions. Plants inoculated with 200 non-surface sterilised nematodes originating from beetles or nematodes cultured for one cycle on fungus showed faster symptom development and higher mortality rates compared to those inoculated with surface sterilized nematodes from beetles or wood logs and cultured for one generation or multiple generations on fungus. Wilt symptoms developed faster on 3-4-year old seedlings of *P. thunbergii* than that on 7-9-year old plants; seedlings were more sensitive than mature plants. *P. taeda* and *P. elliotti* showed highest resistance/tolerance against *B. xylophilus*; *P. massoniana* was less susceptible for *B. xylophilus*. *P. thunbergii* is the most susceptible *Pinus* species. On *P. thunbergii* seedlings, a Canadian isolate (BxCAN) of *B. xylophilu*s caused slower symptom development and lower mortality than four other populations originating from China, Japan and Portugal.

Little is known about the molecular mechanisms underlying *B. xylophilus* pathogenicity and host–parasite interactions. A series of candidate pathogenicity genes had been identified from EST of *B. xylophilus* but nothing was known about variation in these genes. The variation in four pathogenicity related genes (β-1,3-endoglucanase, cellulase, expansin and pectate lyase) was examined for five *B. xylophilus* populations. Although the sequences of each of the four genes between and within nematode populations were very similar to each other with low pairwise divergence values, the population from Canada showed a higher variation when compared to the other four populations. The North American native populations are likely to have a higher genetic variability than the introduced populations.

The limited information obtained in analysing four pathogenicity related genes failed to give a clear signal as to which genes may be important in pathogenicity of *B. xylophilus*. Therefore, an expansin-like gene from EST of *B. xylophilus* was selected for functional analysis because detailed information on this gene was lacking. The cDNA of *Bx-exp-1* gene

is 633 nucleotides long and contains a 513-bp open reading frame (ORF) and encodes a protein of 171 amino acids. The expansin transcripts of *B. xylophilus* were specifically expressed in the oesophageal gland cells as revealed by *in situ* hybridization. The predicted expansin-like protein Bx-EXP-1 from *B. xylophilus* only consisted of an expansin-like domain (Kikuchi *et al.*, 2008) and may assist the rapid penetration of the nematode though plant cells.

No RNAi studies have previously been reported for any migratory plant-parasitic nematodes; a method for RNAi by *B. xylophilus* was developed. Octopamine inhibited uptake of dsRNA by *B. xylophilus.* The proportion of *B. xylophilus* taking up dsRNA was far greater than that of *B. mucronatus.* Two genes, endo-β-1,3-glucanase and Ribosomal Protein Subunit 23 (*rps*23) were selected for RNAi because silencing endo-β-1,3-glucanase leads to a reduction in the efficiency of feeding on fungus while silencing *rps*23 gives a lethal phenotype. The limited success in silencing the genes was obtained when 3 mM spermidine, 0.05% gelatin and lipofectin or lipofectamine were added to the dsRNA-nematode mixture. A phenotype as a result of the RNAi effect was observed in *B. xylophilus* when targeting the *rps23* gene but not in the β-1,3-endoglucanase gene. The conditions for RNAi need to be further modified before RNAi can be used routinely with *B. xylophilus* for gene functional analyses.

Samenvatting

De dennenhoutnematode, *Bursaphelenchus xylophilus*, die door *Monochamus* kevers verspreid wordt, veroorzaakt verwelking van dennen in China, Japan, Zuid-Korea, Taiwan en Portugal maar werd ook gedetecteerd in de Verenigde Staten van Amerika, Canada en Mexico. *B. xylophilus* is afkomstig van Noord-Amerika waar het natuurlijk coniferenbestand resistent is tegen de nematode. De strategie voor de bestrijding van de dennenverwelking omvat verstrengde quarantainemaatregelen, geïntegreerde bestrijding van de insectvectoren, landbouwkundige maatregelen en heraanplanting met resistente *Pinus* soorten.

Bursaphelenchus xylophilus kan verspreid worden over lange afstanden *via* het verpakkingshout van verscheepte goederen. Plantenquarantaine is een afdoend middel om de invoer van *B. xylophilus* van geïnfecteerde gebieden in niet-geïnfecteerde gebieden te vermijden *via* ingevoerd houtmateriaal dat nematoden en/of vectoren bevat. Tijdens mijn deelname aan een quarantaineïnspectie in Nanjing (2003-2004) identificeerde ik vijf gekende en een onbekende *Bursaphelenchus* soorten uit 13 monsters van ingevoerd verpakkingshout afkomstig van acht landen en regio's. De beschreven *Bursaphelenchus* soorten werden geïdentificeerd aan de hand van hun morfologie, morfometrie en ITS-RFLP patronen. Twee monsters bevatten *B. xylophilus*; een uit Canada (de M-vorm) en een ander uit Japan (R-vorm). Vijf monsters werden geïdentificeerd als *B. mucronatus*; drie behoorden tot het Europese genotype en twee tot het Oost-Asiatische genotype. Twee monsters (Duitsland en Japan) weren herkend als *B. rainulfi*; twee monster (Korea en Japan) werden geïdentificeerd als *B. doui* en een monster uit Honkong werd geïdentificeerd als *B. thailandae*. Voor alle geïdentificeerde soorten werden nieuwe gegevens over de morfometrie toegevoegd aan de reeds gekende gegevens. De CDA van de morfometrische gegevens stelde mij in staat de vijf beschreven soorten van elkaar te onderscheiden aan de hand van respectievelijk negen en acht karakteristieken van mannetjes en vrouwtjes. Deze karakteristieken komen gedeeltelijk overeen met de taxonomische meest informatieve karakters voor het geslacht *Bursaphelenchus*, nl. de lichaamslengte, de stekellengte, de lengte van de spicula, en de waarden a, c, en c'. De fylogenitische dendrogrammen, afgeleid werden uit gegevens van de D2D3 sequenties, ondersteunen de opsplitsing tussen de Oost-Asiatische en Europese genotypen van *B. mucronatus. Bursaphelenchus doui* clusterde met *B. xilophilus* en *B. mucronatus*, beide leden van de xylophilus-groep die zich onderscheidt van *B. rainulfi* en *B. thailandae.*

De nieuw beschreven soort, *Bursaphelenchus chengi*, wordt gekarakteriseerd door een middelmatige lichaamslengte voor beide geslachten, het voorkomen van slechts twee groeven in het laterale veld en de robuste en sterk gebogen spicula met een brede en stompe cucullus. De lamina van de spicula is hoekvormig aan het uiteinde, het rostrum digitate en de condylus afgerond. De staart is boogvormig met een puntvormig uiteinde. De bursa is gewoonlijk afgeknot met een ingesneden achterkant in sommige specimen of anders afgerond met een fijne punt op de as. De vrouwtjes hebben een kleine vulvaflap die gevormd is door een korte verlenging van de cuticula van de voorste lip, en een conische staart die zacht toeloopt naar een bijna rechte of lichtjes teruggebogen, gepunte of ronde terminus. Omwille van de aanwezigheid van twee laterale lijnen, de gelijkaardige vorm van de spicula, de toelopende staart bij de vrouwtjes en het voorkomen van een kleine vulvaflap, wordt *B. chengi* samen met *B. abietinus*, *B. antoniae*, *B. hellenicus*, *B. hylobianum* en *B. rainulfi* gegroepeerd bij de *abietinus*-groep *sensu* Braasch. ITS-RFLP patronen ondersteunen de identiteit van de nieuwe soort en de fylogenetische analyse van de sequenties van het 28S rDNA D2/D3 domein plaatst de soort dicht bij *B. antoniae* en andere soorten van de *abientus*-groep.

Bursaphelenchus xylophilus wordt verspreid door kevers op het moment van hun rijpingvoeding of de eiafleg. Het uitkomen van de vector *Monochamus alternatus* en de overdracht van *B. xylophilus* tijdens de rijpingsvoeding op dennetwijgen werd gevolgd in 2004 en 2005 in Nanjing. Het uitkomen begon aan het einde van april en duurde tot het einde van juni; het had een hoogtepunt tussen einde van mei en begin juni. In 2004 en 2005 werden er respectievelijk 438 en 927 volwassen kevers verzameld; ongeveer 70% van de kevers kwam uit tijdens de piekperiode. De visuele schatting van de nematodenvracht van de vectoren door de inspectie van het atrium van het spiracle van het eerste abdomensegment gaf onnauwkeurige resultaten. Het percentage kevers dat geladen was met nematoden schommelde tussen 20 en 30 percent. Zeventig percent van de nematoden kwam na drie dagen extractie vrij uit de keverkadavers. Er was geen wezenlijk verband tussen het geslacht en de levenduur van de kevers en het aantal nematoden dat achterbleef in de kevers. De transmissie van de nematoden naar dennentwijgen startte 10 dagen na het uikomen van de vector. De transmissieperiode kon duren tot 79 dagen na het uitkomen van de kever. Uit het waarnemen van het aantal nematoden dat per dag naar de twijgen werd overgebracht konden twee typen van transmissiecurve worden afgeleid. Het unimodale patroon piekte tussen de derde en zesde week na het uitkomen van de volwassen kevers; het bimodale model

vertoonde twee pieken: de eerste tussen week twee en drie, de andere tussen de vijfde en zevende week na uitkomen van de kevers.

De meest afdoende bestrijding van *B. xylophilus* is de heraanplant met resistente *Pinus* soorten of andere resistentie coniferen. De systemen die gebruikt worden voor het screenen van resistentie hangen af van de aggressiviteit van de gebruikte nematodepopulaties en hun pathogeniciteit voor de testplanten. De variatie die in eerdere screeningstesten werd geobserveerd werd bekritiseerd in de literatuur. Om die reden werden pathogeniciteitsproeven uitgevoerd ten einde de factoren te evalueren die de pathogeniciteit van *B. xylophilus* voor *P. thunbergii* beïnvloeden, maar ook om informatie te evrwerven over de verschillen in pathogeniciteit van verschillende populaties van *B. xylophilus* voor verschillende soorten *Pinus*. Deze experimenten bevatten de simulatie van de natuurlijke infectie door de vectorkever maar ook de kunstmatige inoculatie van juvenilen die geëxtraheerd werden uit kevers.

De variablele resulaten die tijdens de twee jaren van experimenteren werden verkregen, bewezen de onuitvoerbaarheid van de simulatie van de natuurlijke infectie. De kunstmatige inoculatie is de enige praktische methode om de pathogeniciteit van *B. xylophilus* op *Pinus* soorten te onderzoeken; verschillende factoren moeten echter in overweging worden genomen. De inoculumdichtheid beïnvloedt in belangrijke mate de expressie van de pathogeniciteit van *B. xylophilus* op *P. thunbergii*. Tweehonderd nematoden per plant bleek een geschikte dichtheid voor de kunstmatige inoculatie; ze benadert de maximum dichtheid (350) die door een kever per dacht wordt overgebracht in natuurlijke omstandigheden. Planten die geïnoculeerd werden met 200 niet-oppervlakkig gesteriliseerde nematode afkomstig van kevers of van een schimmelkweek, vertoonden een snellere ontwikkeling van de symptomen en een hogere afstervingsgraad dan deze geïnoculeerd met gesteriliseerde nematoden afkomstig van kevers of boomstamen en voor een of meerdere generaties vermeerderd op schimmels. Drie tot vierjarige *P. thunbergii* zaailingen verwelkten vlugger dan 7-9-jaar oude planten; zaailingen waren gevoeliger dan volwassen planten. *P. taeda* en *P. elliotti* vertoonden de grootste resistentie/tolerantie voor *B. xylophilus*. *P. thunbergii* is de meest gevoelige *Pinus* soort. Op *P. thunbergii* zaailingen veroorzaakte een Canadese populatie van *B. xylophilus* (BxCAN) een tragere symptoomontwikkeling en kleinere mortaliteit dan vier andere *B. xylophilus* poplutaties afkomstig uit China, Japan en Portugal.

Er is weinig gekend over de moleculaire mechanismen die aan de grondslag liggen van de pathogeniciteit van *B. xylophilus* en de interacties tussen de waardplant en de parasiet. Eerder werd een reeks kandidaat-pathogeniciteitsgenen geïdentificeerd uit EST van *B.*

xylophilus; niets was evenwel geweten over de variatie binnen deze genen. De variatie in vier van de pathogeniciteitsgerelateerde genen (β-1,3-endoglucanase, cellulase, expansin en pectate lyase) werd onderzocht voor vijf *B. xylophilus* populaties. Niettegenstaande de sequenties van elk van de vier genen tussen en binnen de nematodenpopulaties vrij gelijkaardig waren met lage paarsgewijze afwijkingswaarden, vertoonde de populatie uit Canada, vergeleken met de andere populaties, een grotere variatie. Populaties uit Noord-Amerika hebben waarschijnlijk een grotere genetische variabiliteit dan de geïntroduceerde piopulaties.

De beperkte informatie die verkregen werd met de analyse van vier patogeniciteitsgerelateerde genen kon geen duidelijke indicatie geven over genen die mogelijk belangrijk zijn in de virulentie van *B. xylophilus*. Daarom werd een expansin-achtig gen uit EST van *B. xylophilus* geselecteerd voor functionele analyse omdat gedetailleerde informatie over dit gen ontbrak. De cDNA van het *Bx-exp-1* gen is 633 nucleotiden groot, bevat een 513-bp open reading frame en codeert een eiwit van 171 aminozuren. *In* situ hybridisatie maakte duidelijk dat de expansin-transcripten van *B. xylophilus* specifiek waarneembaar zijn in de slokdarmkliercellen. Het voorspelde expansin-achtige eiwit BX-EXP-1 van *B. xylophilus* bestond alleen uit een expansin-achtig domein (Kikuchi *et al.*, 2008) en zou kunnen bijdragen tot de vlugge penetratie van de nematode in plantencellen.

RNAi studies over migrerende palntenparasiterende nematoden zijn onbestaande. Daarom werd een methode voor RNAi bij *B. xylophilus* ontwikkeld. Octopamine inhibeerde de opname van dsRNA bij *B. xylophilus*. De proportie individuen die dsRNA opnamen was beduidend groter bij *B. xylophilus* dan bij *B. mucronatus*. Twee genen, endo-β-1,3-glucanase en Ribosomal Protein Subunit 23 (*rps*23), werden geselecteerd voor RNAi omdat enerzijds het uitschakelen van endo-β-1,3-glucanase leidt tot een reductie van de efficiêntie van voeden op de schimmel terwijl anderzijds het uitschakelen van *rps*23 een lethaal fenotype oplevert. Een beperkt succes in het uitschakelen van de genen werd verkregen als 3 mM spermidine, 0.05% gelatin en lipofectin of lipofectamine toegeveogd werden aan het dsRNA-nematode mengsel. Een fenotype als resultaat van het RNAi effect werd geobserveerd in *B. xylophilus* als het *rps*23 gen werd geviseerd maar niet bij het β-1,3-endoglucanase gen. De voorwaarden voor RNAi moeten verder gewijzigd worden vooralleer RNAi routinematig gebruikt kan worden voor genfunctionele analyse.

Abad, P. (2000). Satellite DNA used as a species-specific probe for identification of *Bursaphelenchus xylophilus*. *Bulletin OEPP* 30, 571-574.

Abad, P., Tàres, S., Bruguier, N. & Guiran, G. (1991). Characterization of the relationships in the pinewood nematode species complex (PWNSC) (*Bursaphelenchus* spp.) using a heterologous *Unc-22* DNA probe from *Caenorhabditis elegans*. *Parasitology* 102, 303-308.

Abelleira, A., Escuer, M., Arias, M. & Mansilla, J. (2003). The genus *Bursaphelenchus* Fuchs (Nematoda: Aphelenchida) in north-west Spain. *Nematology* 5, 677-685.

Aikawa, T. & Togashi, K. (1997). An effect of inoculum quantity of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) on the nematode load of *Monochamus alternatus* (Coleoptera: Cerambycidae) in laboratory. *Japanese Journal of Nematology* 27, 14-21.

Aikawa, T. & Togashi, K. (2000). Movement of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) in tracheal system of adult *Monochamus alternatus* (Coleoptera: Cerambycidae). *Nematology* 2, 495-500.

Aikawa, T., Maehara, N., Futai, K. & Togashi, K. (1997). A simple method for loading adult *Monochamus alternatus* (Coleoptera: Cerambycidae) with *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *Applied Entomology and Zoology* 32, 341-346.

Akbulut, S., Braasch, H., Baysal, I., Brandstetter, M. & Burgermeister, W. (2007). Description of *Bursaphelenchus anamurius* sp. n. (Nematoda: Parasitaphelenchidae) isolated from pine wood in Turkey. *Nematology* 9, 859-867.

Alkharouf, N.W., Klink, V.P. & Matthews, B.F. (2007). Identification of *Heterodera glycines* (soybean cyst nematode [SCN]) cDNA sequences with high identity to those of *Caenorhabditis elegans* having lethal mutant or RNAi phenotypes. *Experimental Parasitology* 115, 247-258.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zheng, Z., Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389-3402.

Arakawa, Y. & Togashi, K. (2002). Newly discovered transmission pathway of *Bursaphelenchus xylophilus* from males of the beetle *Monochamus alternatus* to *Pinus densiflora* trees via oviposition wounds. *Journal of Nematology* 34, 396-404.

Araújo, J.C.A., Araújo, A.E. & Santos, A.F. (1998). Population dynamics of *Rhynchophorus palmarum* and its association with *Bursaphelenchus cocophilus* on oil palm in the state of Amazonas, Brazil. *Fitopatologia Brasileira* 23, 23-26.

Armstrong, M.R., Whisson, S.C., Pritchard, L., Bos, J.I.B., Venter, E., Avrova, A.O., Rehmany, A.P., Bo¨hme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Fraser, A., Lord, A., Quail, M.A., Churcher, C., Hall, N., Berriman, M., Huang, S. Kamoun, S., Beynon, J.L. & Birch, P.R.J. (2005). An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. *Proceedings of the National Academy of Sciences of USA* 102, 7766-7771.

Bai, X. & Cheng, H. (1993). Possibility of pine wilt disease epidemic in south China. *Journal of Nanjing Agricultural University* 16, 59-62.

Bakhetia, M., Charlton, W.L., Urwin, P.E., McPherson, M.J. & Atkinson, H.J. (2005) RNA interference and plant parasitic nematodes. *Trends in Plant Science* 10, 362-367.

Bakke, A., Anderson, R.V. & Kvamme, T. (1991). Pathogenicity of the nematodes *Bursaphelenchus xylophilus* and *B. mucronatus* to *Pinus sylvestris* seedlings: a greenhouse test. *Scandinavian Journal of Forest Research* 6, 407-412.

Beckenbach, K., Smith, M. & Webster, J. (1992). Taxonomic affinities and intra- and interspecific variation in *Bursaphelenchus* spp. as determined by polymerase chain reaction. *Journal of Nematology* 24, 140-147.

Beckenbach, K., Blaxter, M. & Webster, J.M. (1999). Phylogeny of *Bursaphelenchus* species derived from analysis of ribosomal internal transcribed spacer DNA sequences. *Nematology* 1, 539-548.

Bedker, P.J. & Blanchette, R.A. (1988). Mortality of Scots pine following inoculation with the pinewood nematode *Bursaphelenchus xylophilus*. *Canadian Journal of Forest Research* 18, 574-580.

Bedker, P.J., Wingfield, M.F. & Blanchette, R.A. (1987). Pathogenicity of *Bursaphelenchus xylophilus* on tree species of pine. *Canadian Journal of Forest Research* 17, 51-57.

Bedker, P.J., Wingfield, M.J., Burnes, T.A. & Blanchette, R.A. (1984). Host specificity of the pine wood nematode in the North Central United States and Canada. In: Dropkin, V. (Ed.) *Proceedings of the United States-Japan Seminar: The resistance mechanisms of pines against pine wilt disease.* 7-11 May 1984, East-West Center, Honolulu, Hawaii. National Science Foundation USA. Japan Society for the Promotion of Science, pp. 46-62.

Bergdahl, D.R. (1999). Threat of pine wilt disease to coniferous forests around the world. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 225-229.

Bergdahl, D.R. & Halik, S. (1999). Inoculated *Pinus sylvestris* serve as long-term hosts for *Bursaphelenchus xylophilus.* In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 73-78.

Bolla, R.I. & Jordan, W. (1982). Cultivation of the pine wilt nematode, *Bursaphelenchus xylophilus,* in axenic culture media. *Journal of Nematology 14*, 377-381.

Bolla, R.I. & Wood, R. (1999). Pathogenicity and potential for spread of the pine wood nematode *Bursaphelenchus* spp. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 27-28 October 1998.* Kyoto, Japan, Shokado, Shoten, pp. 3-13.

Bolla, R.I. & Wood, R. (2004). Pinewood nematode: pathogenic or political? In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus*. Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1.* Leiden, The Netherlands, E. J. Brill, pp. 31-54.

Bolla, R.I., Shaheen, F. & Winters, R.E.K. (1982). Phytotoxin production in *Bursaphelenchus xylophilus* pine wilt. *Journal of Nematology* 14, 431.

Bolla, R.I., Shaheen, F. & Winter, R.E.K. (1984). Phytotoxins in *Bursaphelenchus xylophilus* induced pine wilt. *Proceedings of the United States-Japan Seminar: The resistance mechanisms of pines against pine wilt disease,* 7-11 May 1984, East-West Center, Honolulu, Hawaii. pp. 119-126.

Bolla, R.I., Weaver, C. & Winter, R. (1988). Genomic differences among pathotypes of *Bursaphelenchus xylophilus*. *Journal of Nematology* 20, 309-316.

Bolla, R.I., Winter, R.E.K., Fitzsimmons, K. & Linit, M.J. (1986). Pathotypes of the pinewood nematode *Bursaphelenchus xylophilus*. *Journal of Nematology* 18, 230-238.

Bowers, W.W., Hudak, J. & Raske, A.G. (1992). Host and vector surveys for the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Nematoda: Aphelenchoididae) in Canada. *Information Report Newfoundland and Labrador Region, Forestry Canada* (N-X-285), 55 pp.

Braasch, H. (1996). Pathogenicity tests with *Bursaphelenchus mucronatus* on Scots pine and Norway spruce seedlings in Germany. *European Journal of Forest Pathology* 26, 205-216.

Braasch, H. (1997). Host and pathogenicity tests with pine wood nematode (*Bursaphelenchus xylophilus*) from North America under Central European weather conditions. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 49, 209-214.

Braasch, H. (2000a). Influence of temperature and water supply on mortality of 3-year-old pines inoculated with *Bursaphelenchus xylophilus* and *B. mucronatus*. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 52, 244-249.

Braasch, H. (2000b). Quarantine pest pine wood nematode introduced into Portugal. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 52, 21-22.

Braasch, H. (2000c). *Bursaphelenchus paracorneolus* sp. nov. (Nematoda, Parasitaphelenchidae) from coniferous wood in Germany and remarks on its biology and distribution. *Annales Zoologici* 50, 177-182.

Braasch, H. (2001). *Bursaphelenchus* species in conifers in Europe: Distribution and morphological relationships. *EPPO Bulletin* 31, 127-142.

Braasch, H. (2004a). Morphology of *Bursaphelenchus xylophilus* compared with other *Bursaphelenchus* species. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus. *Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1*. Leiden, The Netherlands, E. J. Brill, pp. 127-143.

Braasch, H. (2004b). A new *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) sharing characters with Ektaphelenchidae from the People's Republic of China. *Zootaxa* 624, 1-10.

Braasch, H. & Braasch-Bidasak, R. (2002). First record of the genus *Bursaphelenchus* Fuchs, 1937 in Thailand and description of *B. thailandae* sp. n. (Nematoda: Parasitaphelenchidae). *Nematology* 4, 853-863.

Braasch, H. & Burgermeister, W. (2002). *Bursaphelenchus rainulfi* sp. n. (Nematoda: Parasitaphelenchidae), first record of the genus *Bursaphelenchus* Fuchs, 1937 from Malaysia. *Nematology* 4, 971-978.

Braasch, H. & Philis, J. (2002). New records of *Bursaphelenchus* spp. in Cyprus. *Nematologia Mediterranea* 30, 55-57.

Braasch, H. & Schmutzenhofer, H. (2000). *Bursaphelenchus abietinus* sp. n. (Nematoda, Parasitaphelenchidae). *Russian Journal of Nematology* 8, 1-6.

Braasch, H., Burgermeister, W. & Pastrik, K.H. (1995). Differentiation of three *Bursaphelenchus* species by means of RAPD-PCR. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 47, 310-314.

Braasch, H., Burgermeister, W & Hoyer, U. (1998). Diversity within the species *Bursaphelenchus mucronatus* worldwide. *Nematologica* 44, 465-466.

Braasch, H., Gu, J., Burgermeister, W. & Zhang, J. (2004a). *Bursaphelenchus doui* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Taiwan and South Korea: a new species of the xylophilus group. *Russian Journal of Nematology* 12, 19-27.

Braasch, H., Shonfeld, U., Polomski, J. & Burgermeister, W. (2004b). *Bursaphelenchus vallesianus* sp. n.: a new species of the *Bursaphelenchus sexdentati* group. *Nematologia Mediterranea* 32, 71-79.

Braasch, H., Burgermeister, W., Brandstetter, M. & Metge, K. (2007a). *Bursaphelenchus africanus* sp. n. (Nematoda: Parasitaphelenchidae) found in packaging wood exported from South Africa to Ningbo/China. *Journal of nematode morphology and systematics* 9, 71-81.

Braasch, H., Gu J. & Brandstetter, M. (2007b). *Bursaphelenchus burgermeisteri* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Japan: a second species of the "africanus" group. *Journal of nematode morphology and systematics* 10, 39-48.

Braasch, H., Burgermeister, W., Harmey, M.A., Michalopoulos-Skarmoutsos, H., Tomiczek, C. & Caroppo, S. (2000). Pest risk analysis of pinewood nematode related *Bursaphelenchus* species in view of South European pine wilt and wood imports from Asia. *Final Report of EU Research Project, Fair CT 95-0083*, 251 pp.

Braasch, H., Tomiczek, C., Metge, K., Hoyer, U., Burgermeister, W., Wulfert, I. & Schönfeld, U. (2001). Records of *Bursaphelenchus* spp. (Nematoda, Parasitaphelenchidae) in coniferous timber imported from the Asian part of Russia. *Forest Pathology* 31, 129-140.

Braasch, H., Burgermeister, W., Schönfeld, U., Metge, K. & Brandstetter, M. (2006). *Bursaphelenchus hildegardae* sp. n. (Nematoda: Parasitaphelenchidae) - a new species belonging to the 'eggersi' group. *Journal of nematode morphology and systematics* 9, 27-38.

Brammer, A.S. & Crow, W.T. (2001). *Red ring nematode,* Bursaphelenchus cocophilus *(Cobb) Baujard (Nematoda: Secernentea: Tylenchida: Aphelenchina: Aphelenchoidea: Bursaphelenchina) formerly* Rhadinaphelenchus cocophilus. EENY-236, Entomology and Nematology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, pp. 1-4.

Britton, K.O. & Sun, J.H. (2002)**.** Unwelcome Guests: Exotic Forest Pests. *Acta Entomologica Sinica* 45, 121-130.

Burgermeister, W, Gu., J. & Braasch, H. (2005a). *Bursaphelenchus arthuri* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Taiwan and South Korea - a new species belonging to the fungivorus group. *Journal of nematode morphology and systematics* 8, 7-18.

Burgermeister, W., Metge, K., Braasch, H. & Buchbach, E. (2005b). ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) and observations on their distribution. *Russian Journal of Nematology* 13, 29-42.

Burnes, T.A., Wingfield, M.J., Baker, F.A., Knowles, K. & Beaubien, Y. (1985). The pine wood nematode in jack pine infected with dwarf mistletoe. *Forest Science* 31, 866-870.

Cao, Y., Han, Z. & Li, C. (2001). Studies on wilting toxic substances produced in pines infected by pine wood nematodes. *Scientia Silvae Sinicae* 37, 75-79.

Cao, Y., Ma, H., Yang, W., Bai, G., Li, H., Hu, J. & Wang, Y. (2005). Development and application of immunomagnetic separation ELISA for identification of *Bursaphelenchus xylophilus*. *Forest Research* 18, 585-589.

Caroppo, S., Cavalli, M., Coniglio, D. & Ambrogioni, L. (2000). Pathogenicity studies with various Bursaphelenchus populations on conifer seedlings under controlled and open air conditions. *Redia* 83, 61-75.

Castagnone, C., Abad, P. & Castagnone-Sereno, P. (2005). Satellite DNA-based species specific identification of single individuals of the pinewood nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *European Journal of Plant Pathology* 112, 191-193.

Chai, X., Zhang, D., Zhang, G., Luo, J. & Zhou, G. (2000). The number of *Bursaphelenchus xylophilus* carried by *Monochamus alternatus* adults. *Journal of Northeast Forestry University* 28, 99-101

Chang, R., Chao, J., Fan, Y., Lu, S. & Jou, W. (1995). Investigation the insect vector of pine wilt disease in Taiwan. *Plant Protection Bulletin* 37, 448 (Abstr).

Chen, Q. Rehman, S., Smant, G. & Jones J.T. (2005). Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi. *Molecular Plant-Microbe Interaction* 18, 621-625.

Cheng, H., Lin, M., Li, W. & Fang, Z. (1983). The occurrence of a pine wilting disease caused by a nematode found in Nanjing. *Forest Pest and Disease*, 1-5.

Cobb, N.A. (1919). A newly discovered nematode (*Aphelenchus cocophilus* n. sp.) connected with a serious disease of the coconut palm. *West Indian Bulletin* 17, 203-210.

Coomans, A., De Coninck, L. & Heip, C. (1978). Data to be considered in descriptions of new species or redescriptions of poorly known species. *Annales de la Société Royale Zoologique de Belgique* 108, 119-122.

Coomans, A. (2002). Present status and future of nematode systematic. *Nematology* 4, 573-582.

Cosgrove, D.J. (2000). Loosening of plant cell walls by expansins. *Nature* 407, 321-326.

Dan, Y. & Yu, S. (2003). Identification of *Bursaphelenchus* spp. on pine wood in Yunnan Province. *Acta Phytopathologica Sinica* 33, 401-405.

Dautova, M., Rosso, M., Abad, P., Gommers, F.J., Bakker, J. & Smant, G. (2001). Single pass cDNA sequencing: a powerful tool to analyse gene expression in preparasitic juveniles of the southern root-knot nematode *Meloidogyne incognita. Nematology* 3, 129-139.

Davis, E.L., Hussey, R.S., Baum, T.J., Bakker, J., Schots, A., Rosso, M.N. & Abad, P. (2000). Nematode parasitism genes. *Annual Review of Phytopathology* 38, 365-396.

De Boer, J.M., Yan, Y., Smant, G., Davis, E.L. & Baum, T.J. (1998). *In-situ* hybridization to messenger RNA in *Heterodera glycines*. *Journal of Nematology* 30, 309-312.

De Boer, J., Yan, Y., Wang, X., Smant, G., Hussey, R.S., Davis, E.L. & Baum, T.J. (1999). Developmental expression of secretory beta-1,4-endoglucanases in the subventral oesophageal glands of *Heterodera glycines*. *Molecular Plant-Microbe Interaction* 12, 663-669.

De Boer, J.M., McDermott, J.P., Davis, E.L., Hussey, R.S., Popeijus, H., Smant, G. & Baum, T.J. (2002). Cloning of a putative pectate lyase gene expressed in the subventral Oesophageal glands of *Heterodera glycines. Journal of Nematology* 34, 9-11.

De Grisse, A.T. (1969). Redescription ou modifications de quelques techniques utilisées dans l'étude de nématodes phytoparasitaires. *Medelelingen Rijksfaculteit Landbouwwetenschappe*n *Gent* 34, 315-359.

De Ley, P., Félix M.A., Frisse, L.M., Nadler, S.A., Sternberg, P.W. & Thomas, W.K. (1999). Molecular and morphological characterisation of two reproductively isolated species with mirror-image anatomy (Nematoda: Cephalobidae). *Nematology* 2, 591-612.

De Ley, P. & Blaxter, M. (2004). A new system for Nematoda: combining morphological characters with molecular trees, and translation clades into ranks and taxa. *Nematology Monographs and perspectives* 2, 633-653.

Dean, C.G. (1979). *Red ring disease of* Cocos nucifera *L. caused* by Rhadinaphelenchus cocophilus (*Cobb, 1919) Goodey, 1960. An annotated bibliography and review*. Technical Communication No. 47, Farnham Royal, UK, Commonwealth Agricultural Bureaux, 70 pp.

Ding, X., Shields, J., Allen, R. & Hussey, R.S. (1998). A secretory cellulose-binding protein cDNA cloned from the root-knot nematode (*Meloidogyne incognita*). *Molecular Plant-Microbe Interaction* 11, 952-959.

Doyle, E.A., & Lambert, K.N. (2002). Cloning and characterization of an oesophageal-gland-specific pectate lyase from the root-knot nematode *Meloidogyne javanica*. *Molecular Plant-Microbe Interaction* 15, 549-556.

Dropkin, V.H. & Foudin, A.S. (1979). Report of the occurrence of *Bursaphelenchus lignicolus* induced pine wilt disease in Missouri. *Plant Disease Reporter* 63, 904-905.

Dropkin, V.H. & Linit, M. (1982). Pine wilt - a disease you should know. *Journal of Arboriculture* 8, 1-6.

Duncan, L.W. & Moens, M. (2006). Migratory endoparasitic nematodes. In: Perry, R.N. & Moens, M. (Eds) Plant nematology. CABI, Wallingford, UK. pp. 123-152.

Dwinell, L.D. (1984). Relative susceptibilities of five south-eastern pine species to the pinewood nematode, *Bursaphelenchus xylophilus*. *Phytopathology* 74, 870.

Dwinell, L.D. (1985). Relative susceptibilities of five pine species to three populations of the pinewood nematode. *Plant Disease* 69, 440-442.

Dwinell, L.D. (1993). First report of pinewood nematode (*Bursaphelenchus xylophilus*) in Mexico. *Plant Disease* 77, 846 [Abstr.].

Edwards, O.R. & Linit, M.J. (1992). Transmission of *Bursaphelenchus xylophilus* through oviposition wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). *Journal of Nematology* 24, 133-139.

Eisenback, J. (1985). Techniques for preparing nematodes for scanning electron microscopy. In: Barker, K.R., Carter, C.C. & Sasser, N.S. (Eds). *An advanced treatise on* Meloidogyne*, Volume II.* Raleigh, NC, USA, North Carolina State University Graphics, pp. 79-105.

Enda, N. (1994). Japanese pine sawyer. In: Kobayashi, F. & Taketani, A. (Eds). *Forest pest.* Tokyo, Japan, Yokendo, pp. 149-153.

EPPO (1986). EPPO data sheets on quarantine organisms. List A1, No. 158 - *Bursaphelenchus xylophilus* (Steiner & Bührer) Nickle *et al.* Nematoda: Aphelenchoididae. *EPPO Bulletin* 16, 55-60.

Esser, R.P. & Meredith, J.A. (1987). *Red ring nematode*. Nematology Circular No. 14, Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville.

Evans, H.F., McNamara, D.G., Braasch, H., Cha-Doeuf, J. & Magnusson, C. (1996). Pest Risk Analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus *Monochamus*. *EPPO Bulletin* 26, 199-249.

Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C.J, Hofmann, K. & Bairoch, A. (2002). The Prosite database, its status in 2002. *Nucleic Acids Research* 30, 235-238.

Fanelli, E., Di Vito, M., Jones, J.T. & De Giorgi, C. (2005). Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia,* using RNAi. *Gene* 349, 87-95.

Fang, Y., Zhao, J. & Zhuo, K. (2002a). Description of *Bursaphelenchus dongguanensis* sp. n. in China (Nematoda: Aphelenchoididae). *Journal of Huazhong Agricultural University* 21, 109-111.

Fang, Y., Zhuo, K. & Zhao, J. (2002b). Description of *Bursaphelenchus aberrans* n. sp. (Nematoda: Parasitaphelenchidae) isolated from pine wood in Guangdong Province, China. *Nematology* 4, 791-794.

Fang, Z. (1998). Methodology of Plant Pathology. 3rd Ed. Beijing, China, Agricultural Publisher of China, 427 pp.

Fauziah, B.A., Hidaka, T. & Tabata, K. (1987). The reproductive behaviour of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae). *Applied Entomology and Zoology* 22, 272-285.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783-791.

Ferris, V.R., Ferris, J.M. & Faghihi, J. (1993). Variation in spacer ribosomal DNA in some cyst forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* 16, 177-184.

Fiedling, N.J. & Evans, H.F. (1996). The pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (= *B. lignicolus* Mamiya & Kiyohara): an assessment of the current position. *Forestry (Oxford)* 69, 35-46.

Fire, A., Xu, S., Montgomery, M.K., Kostsas, S.A., Driver, S.E. & Mello, C.C. (1998). Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-811.

Fonseca, L., Curtis, R., Halsey, K., Santos, M. Abrantes, M. & Santos, M. (2006). Morphological, molecular and serological characterization of *Bursaphelenchus xylophilus* isolates. *Pine wilt disease: a worldwide threat to forest ecosystems, International symposium. 10-14 July, Lisbon:* 63-64 (Abst.).

François, C., Castagnone, C., Boonham, N., Tomlinson, J., Lawson, R., Hockland, S., Quill, J., Vieira, P., Mota, M. & Castagnone-Sereno, P. (2007). Satellite DNA as a target for TaqMan real-time PCR detection of the pinewood nematode, *Bursaphelenchus xylophilus. Molecular Plant Pathology* 8, 803-809.

Fuchs, A.G. (1937). Neue parasitische und halbparasitische Nematoden bei Borkenkäfern und einige andere Nematoden. I. Teil die Parasiten der Waldgartner *Myelophilus piniperda* L. und *minor* Hartig und die Genera *Rhabditis* Dujardin, 1845 und *Aphelenchus* Bastian, 1865. *Zoologische Jahrbücher, Abteilung für Systematik Oekologie und Geographie der Tiere, Jena* 70, 291-380.

Fukuda, K., Hogetsu, T. & Suzuki, K. (1994). Ethylene production during symptom development of pine-wilt disease. *European Journal of Forest Pathology* 24, 193-202.

Fukushige, H. (1990). The number of *Bursaphelenchus xylophilus* carried by *Monochamus alternatus* and some possible factors regulating the number. *Japanese Journal of Nematology* 20, 18-24.

Fukushige, H. (1991). Propagation of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) on fungi growing in pine [*Pinus*]-shoot segments. *Applied Entomology and Zoology* 26, 371-376.

Furuno, T. (1982). Studies on the insect damage upon the pine-species imported in Japan (No. 7). On the withering of the pines by the pine wilt. *Kyoto University Forests bulletin* 54, 16-30.

Futai, K. (1980). Developmental rate and population-growth of *Bursaphelenchus lignicolus* (Nematoda, Aphelenchoididae) and *Bursaphelenchus mucronatus*. *Applied Entomology & Zoology* 15, 115-122.

Futai, K. & Furuno, T. (1979). The variety of resistances among pine-species to pine wood nematode, *Bursaphelenchus lignicolus*. *Bulletin of the Kyoto University Forests* 51, 23-36.

Futai, K. & Harashima, S. (1990). Effect of simulated acid mist on pine wilt disease. *Journal of the Japanese Forestry Society* 72, 520-523.

Gao, B., Allen, R., Maier, T., Davis, E.L., Baum, T.J. & Hussey, R.S. (2002). Identification of a new β-1,4-endoglucanase gene expressed in the oesophageal subventral gland cells of *Heterodera glycines*. *Journal of Nematology* 34, 12-15.

Gao, B., Allen, R., Davis, E.L., Baum, T.J. & Hussey, R.S. (2004). Molecular characterisation and developmental expression of a cellulose binding protein gene in the soybean cyst nematode *Heterodera glycines*. *International Journal for Parasitology* 34, 1377-1383.

Ge, J. & Cheng, H. (1993). Study on the resistance of three conifers to pine wood nematode. *Plant Quarantine* 7, 337.

Ge, M. & Xu, F. (1999). Studies on the relationship of occurrence of pine wood nematode (PWN) disease with environment and its control strategies. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 174-177.

Gerber, K. & Giblin-Davis, R.M. (1990). Association of the red ring nematode and other nematode species with the palm weevil, *Rhynchophorus palmarum*. *Journal of Nematology* 22, 143-149.

Gheysen, G. & Vanholme, B. (2007). RNAi from plants to nematodes. *Trends in Biotechnology* 25, 89-92.

Giblin, R.M. & Kaya, H.K. (1983). *Bursaphelenchus seani* n. sp. (Nematoda: Aphelenchoididae), a phoretic associate of *Anthophora bomboides stanfordiana* Cockerell, 1904 (Hymenoptera: Anthophoridae). *Revue de Nématologie* 6, 39-50.

Giblin-Davis, R.M. (1993). Interactions of nematodes with insects. In: Wajid, M. (Ed.). *Nematode interactions*. New York, USA, Chapman & Hall, pp. 302-304.

Giblin-Davis, R.M., Hazir, S., Center, B.J., Ye, W., Keskin, N., Thorp, R.W. & Thomas, W.K. (2005). *Bursaphelenchus anatolius* n. sp (Nematoda: Parasitaphelenchidae), an associate of bees in the genus *Halictus*. *Journal of Nematology* 37, 336-342.

Giblin-Davis, R.M., Kanzaki, N., Ye, W., Center, B.J. & Thomas, W.K. (2006a). Morphology and systematics of *Bursaphelenchus gerberae* n. sp. (Nematoda: Parasitaphelenchidae), a rare associate of the palm weevil, *Rhynchophorus palmarum* in Trinidad. *Zootaxa* 1189, 39-53.

Giblin-Davis, R.M., Kanzaki, N., Ye, W., Mundo-Ocampo, M., Baldwin, J.G. & Thomas, K.W. (2006b). Morphology and description of *Bursaphelenchus platzeri* n. sp (Nematoda: Parasitaphelenchidae), an associate of nitidulid beetles. *Journal of Nematology* 38, 150-157.

Gill, S.C. & von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Annals of Biochemistry* 182, 319-26.

Goellner, M., Wang, X., & Davis, E.L. (2001). Endo-β-1,4-glucanase expression in compatible plant-nematode interactions. *Plant Cell* 13, 2241-2255.

Griffith, R. & Koshy, P.K. (1990). Nematode parasites of coconut and other palms. In: Luc, M., Sikora, R.A. & Bridge, J. (Eds). *Plant-parasitic nematodes in subtropical and tropical agriculture*. Wallingford, UK, CABI Publishing, pp. 363-386.

Grishok, A., Pasquinelli, A., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D., Fire, A., Ruvkun, G. & Mello, C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* 106, 23-34.

Gu, J., Zhang, J., Braasch, H. & Burgermeister, W. (2005). *Bursaphelenchus singaporensis* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Singapore - a new species of the *B. xylophilus* group. *Zootaxa* 988, 1-12.

Gu, J., Braasch, H., Burgermeister, W., & Zhang, J. (2006a) Records of *Bursaphelenchus* spp. intercepted in imported packaging wood at Ningbo, China. *Forest Pathology* 36, 323-333.

Gu, J., Braasch, H., Burgermeister, W., Brandstetter, M. & Zhang, J. (2006b). Description of *Bursaphelenchus yongensis* sp. n. (Nematoda: Parasitaphelenchidae) isolated from Pinus massoniana in China. *Russian Journal of Nematology* 14, 91-99.

Guiran, G. & Bruguier, N. (1989). Hybridization and phylogeny of the pine wood nematode (*Bursaphelenchus* spp.). *Nematologica* 35, 321-330.

Guiran, G., Lee, M., Dalmasso, A. & Bongiovanni, M. (1985). Preliminary attempt to differentiate pinewood nematodes (*Bursaphelenchus* spp.) by enzyme electrophoresis. *Revue de Nématologie* 8, 88-90.

Halik, S. & Bergdahl, D.R. (1994). Long-term survival of *Bursaphelenchus xylophilus* in living *Pinus sylvestris* in an established plantation. *European Journal of Forest Pathology* 24, 357-363.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows95/98/NT. *Nucleic Acids Symposium Series* 41, 95-98.

Han, Z., Hong, Y. & Zhao, B. (2003). A study on pathogenicity of bacteria carried by pine wood nematodes. *Journal of Phytopathology* 151, 683-689.

Hara, N. & Futai, K. (2001). Histological changes in xylem parenchyma cells and the effects on tracheids of Japanese black pine inoculated with pine wood nematode, *Bursaphelenchus xylophilus*. *Journal of the Japanese Forestry Society* 83, 285-289.

Harmey, J. & Harmey, M. (1993). Detection and identification of *Bursaphelenchus* species with DNA fingerprinting and polymerase chain reaction. *Journal of Nematology* 25, 406-415.

Harmey, J.H. & Harmey, M.A. (1994). DNA profiling of *Bursaphelenchus* species. *Gene* 145, 227-230.

Harrison, N.A. & Jones, P. (2003). Diseases of coconut. In: Ploetz, R.C. (Ed.). *Diseases of tropical fruit crops*. Wallingford, UK, CABI Publishing, pp. 197-225.

He, Y., Li, H., Brown, D.J.F., Lamberti, F. & Moens, M. (2003). Isolation and characterisation of microsatellites for *Xiphinema index* using degenerate oligonucleotide primed PCR. *Nematology* 5, 809-819.

Higgins, D.F., Briarty, D.M. & Harmey, M.A. (1999a). Detection of a chitinase gene fragment in Bursaphelenchus species by polymerase chain reaction amplification. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 29-34.

Higgins, D.F., Harmey, M.A. & Jones, D.L. (1999b). Pathogenicity related gene expression in *Bursaphelenchus xylophilus*. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 23-28.

Hong, Y., Cao, Y., Zhao, B. & Han, Z. (2002). Studies on identification and toxicity of bacteria carried by pine wood nematodes. *Journal of Nanjing Forestry University* 26, 37-40.

Hong, Y., Zhao, B., Cao, Y. & Han, Z. (2003). Pathogenicity of bacteria carried by pine wood nematodes. *Journal of Nanjing Forestry University* 27, 45-48.

Hotchkin, P. & Giblin, R. (1984). Comparison of electrophoregrams from *Bursaphelenchus* spp. (Aphelenchoididae). *Revue de Nématologie* 7, 319-320.

Hoyer, U., Burgermeister, W. & Braasch, H. (1998). Identification of *Bursaphelenchus* species (Nematoda: Aphelenchoididae) on the basis of amplified ribosomal DNA (ITS-RFLP). *Nachrichtenblatt für den Deutschen Pfianzenschutzdienst* 50, 273-277.

Hu, K. & Yang, B. (1995). Preliminary comparison of *Bursaphelenchus xylophilus* and *B. mucronatus* utilizing enzyme electrophoresis. *Forest Research* 8, 73-77.

Hu, K., Wang, Q. & Yang, B. (1994). Pathogenic comparison among 14 isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* collected from different regions and countries. *Forest Research* 7, 381-385.

Huang, G., Dong, R.H., Allen, R., Davis, E.L., Baum, T.J. & Hussey, R.S. (2006). A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. *Molecular Plant-Microbe Interactions* 19, 463-470.

Hunt, D.J. (1993). *Aphelenchida, Longidoridae and Trichodoridae: Their systematics and bionomics*. Wallingford, UK, CABI Publishing, 352 pp.

Ichihara, Y., Fukuda, K. & Suzuki, K. (2000). Early symptom development and histological changes associated with migration of *Bursaphelenchus xylophilus* in seedling tissues of *Pinus thunbergii*. *Plant Disease* 84, 675-680.

Ikeda, T. (1984). Integrated pest management of Japanese pine wilt disease. *European Journal of Forest Pathology* 14, 398-414.

Ikeda, T. (1996). Xylem dysfunction in *Bursaphelenchus xylophilus*-infected Pinus thunbergii in relation to xylem cavitation and water status. *Annals of the Phytopathological Society of Japan* 62, 554-558.

Ikeda, T. & Suzaki, T. (1984). Influence of pine-wood nematodes on hydraulic conductivity and water status in *Pinus thunbergii*. *Journal of the Japanese Forestry Society* 66, 412-420.

Ikeda, T. & Kiyohara, T. (1995). Water relations, xylem embolism and histological features of *Pinus thunbergii* inoculated with virulent or avirulent pine wood nematode, *Bursaphelenchus xylophilus*. *Journal of Experimental Botany* 46, 441-449.

Ikeda, T., Mamiya, Y. & Shoji, T. (1989). Anatomical observation of bordered pits in xylem of *Bursaphelenchus xylophilus*-inoculated *Pinus densiflora* shoot cuttings treated with benzoic acid. *Annals of the Phytopathological Society of Japan* 55, 665-666.

Ikeda, T., Kiyohara, T. & Kusunoki, M. (1990). Change in water status of *Pinus thunbergii* Parl. inoculated with species of *Bursaphelenchus*. *Journal of Nematology* 22, 132-135.

Irdani, T., Marinari, A., Bogani, P., Ambrogioni, L., Caroppo, S. & Buiatti, M. (1995). Molecular diversity among pine wood *Bursaphelenchus* populations detected by RAPD analysis. *Redia* 78, 149-161.

Ishida, K., Hogetsu, T., Fukuda, K. & Suzuki, K. (1993). Cortical responses in Japanese black pine to attack by the pine wood nematode. *Canadian Journal of Botany* 71, 1399-1405.

Iwahori, H. & Futai, K. (1995). Comparative movement speed of pathogenic and nonpathogenic isolates of *Bursaphelenchus* nematodes. *Applied Entomology and Zoology* 30, 159-167.

Iwahori, H., Kanzaki, N. & Futai, K. (2004). *Bursaphelenchus xylophilus* and *B. mucronatus* in Japan. In: Cook, R. & Hunt, D.J. (Eds). *Proceedings of the Fourth International Congress of Nematology, 8-13 June 2002, Tenerife, Spain. Nematology monographs and perspectives, Volume 2.* Leiden, The Netherlands, E. J. Brill, pp. 793-803.

Iwahori, H., Tsuda, K., Kanzaki, N., Izui, K. & Futai, K. (1998). PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. *Fundamental and Applied Nematology* 21, 655-666.

Jaubert, S., Ledger, T.N., Laffaire, J. B., Piotte, C., Abad, P., & Rosso, M.N. (2002a). Direct identification of stylet secreted proteins from root-knot nematodes by a proteomic approach. *Molecular and Biochemical Parasitology* 121, 205-211.

Jaubert, S., Laffaire, J.B., Abad, P., & Rosso, M.N. (2002b). A polygalacturonase of animal origin isolated from the root-knot nematode *Meloidogyne incognita*. *FEBS Letters* 522, 109-112.

Jiang, F., Wang, P., Tang, Y., Zhao, F., Xu, Y. & Lu, H. (1998). Prevention and control of *Monochamus alternatus* with attractants. *Journal of Zhejiang Forestry Science and Technology* 18, 23-26.

Jiang, L. & Wang, X. (1995). Qualitative inspection on cellulase in extract and secretion of *Bursaphelenchus xylophilus*. *Forest Pest and Disease*, 9-11.

Jiang, L., Zheng, J., Chen, Z. & Wu, J. (2006). Screening and characterization of monoclonal antibody to *Bursaphelenchus xylophilus*. *Journal of Agricultural Biotechnology* 14, 412-415.

Jiang, P., Wu, Z., Chai, X., He, Z. & Lin, F. (2002). Transmission characteristic of *Bursaphelenchus xylophilus* through *Monochamus alternatus*. *Journal of Nanjing Forestry University* 26, 69-71.

Jiao, G., Shen, P. & Li, H. (1996). Virulence studies of pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle (Tylenchida: Aphelenchoididae) from Japan and Nanjing, China on *Cedrus deodara* and *Pinus massoniana. Plant Quarantine* 10, 193-195.

Jikumru, S. & Togashi, K. (1995). A weak deleterious effect of the avirulent pinewood nematode, *Bursaphelenchus mucronatus* (Nematoda, Aphelenchoididae), (Coleoptera, Cerambycidae). *Applied Entomology and Zoology* 30, 9-16.

Jikumaru, S. & Togashi, K. (2001). Transmission of *Bursaphelenchus mucronatus* (Nematoda: Aphelenchoididae) through feeding wounds by *Monochamus saltuarius* (Coleoptera: Cerambycidae). *Nematology* 3, 325-333.

Jones, J.T., Furlanetto, C. & Kikuchi, T. (2005). Horizontal gene transfer from bacteria and fungi as a driving force in the evolution of plant parasitism in nematodes. *Nematology* 7, 641-646.

Jones, J.T., Furlanetto, C., Bakker, E., Banks, D.A., Blok, V.C., Chen, Q., Phillips, M. S. & Prior, A. (2003). Characterisation of a chorismate mutase from the potato cyst nematode *Globodera pallida*. *Molecular Plant Pathology* 4, 43-50.

Jones, J.T., Moens, M., Mota, M., Li, H. & Kikuchi, T. (2008). *Bursaphelenchus xylophilus:* opportunities in comparative genomics and molecular host-parasite interactions. *Molecular Plant Pathology*. Online available.

Kaneko, S. & Zinno, Y. (1986). Development under different light sources of pine-wilt disease caused by *Bursaphelenchus xylophilus* on the seedlings of Japanese red pine. *Journal of the Japanese Forestry Society* 68, 208-209.

Kang, J.S., Choi. K.S., Shin, S.H., Moon, I.S., Lee, S.G. & Lee, S.H. (2004). Development of an efficient PCR-based diagnostic protocol for the identification of the pinewood nematode *Bursaphelenchus xylophilus* (Nematoda. Parasitaphelenchidae). *Nematology* 6, 279-285.

Kanzaki, N. & Futai, K. (2002a) A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the *xylophilus* group. *Nematology* 4, 35-41.

Kanzaki, N. & Futai, K. (2002b). Phylogenetic analysis of the phoretic association between *Bursaphelenchus conicaudatus* (Nematoda: Aphelenchoididae) and *Psacothea hilaris* (Coleoptera: Cerambycidae). *Nematology* 6, 759-771.

Kanzaki, N. & Futai, K. (2003). Description and phylogeny of *Bursaphelenchus luxuriosae* n. sp. (Nematoda: Aphelenchoididae) isolated from *Acalolepta luxuriosa* (Coleoptera: Cerambycidae). *Nematology* 5, 565-572.

Kanzaki, N., Tsuda, K. & Futai, K. (2000). Description of *Bursaphelenchus conicaudatus* n. sp. (Nematoda: Aphelenchoididae), isolated from the yellow-spotted longicorn beetle, *Psacothea hilaris* (Coleoptera: Cerambycidae) and fig trees, *Ficus carica*. *Nematology* 2, 165-168.

Kasuya, S., Sakura, T. & Kishi, Y. (1990). Selective breeding of resistant pines against pine wood nematode: resistance of the seedlings and the graftings from resistant mother trees. *Bulletin of the Tokyo University Forests*, 19-30.

Kawaguchi, E., Gyokusen, K. & Saito, A. (1999). Behaviour of *Bursaphelenchus xylophilus* and the development of pine wilt disease under shaded condition. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 39-41.

Kawazu, K. & Kaneko, N. (1997). Asepsis of the pine wood nematode isolate OKD-3 causes it to lose its pathogenicity. *Japanese Journal of Nematology* 27, 76-80.

Kawazu, K., Kaneko, N. & Kanzaki, H. (1999). What factors govern the pathogenicity of the pine wood nematode, *Bursaphelenchus xylophilus*? In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 42-46

Kawazu, K., Yamashita, H., Kobayashi, A. & Kanzaki, H. (1998). Isolation of pine-wilting bacteria accompanying pine wood nematode, *Bursaphelenchus xylophilus*, and their toxic metabolites. *Scientific Reports of the Faculty of Agriculture,* Okayama University 1-7.

Kawazu, K., Zhang, H. & Kanzaki, H. (1996a). Accumulation of benzoic acid in suspension cultured cells of *Pinus thunbergii* Parl. in response to phenylacetic acid administration. *Bioscience, Biotechnology and Biochemistry* 60, 1410-1412.

Kawazu, K., Zhang, H., Yamashita, H. & Kanzaki, H. (1996b). Relationship between the pathogenicity of the pine wood nematode, *Bursaphelenchus xylophilus*, and phenylacetic acid production. *Bioscience, Biotechnology and Biochemistry* 60, 1413-1415.

Kikuchi, T., Shibuya, H. & Jones, J.T. (2005). Molecular and biochemical characterization of an endo-β-1,3-glucanase from the pine wood nematode *Bursaphelenchus xylophilus. Biochemical Journal* 389, 117-125.

Kikuchi, T., Jones, J.T., Aikawa, T., Kosaka, H. & Ogura, N. (2004). A family of GHF45 cellulases from the pine wood nematode *Bursaphelenchus xylophilus*. *FEBS Letters* 572, 201-205.

Kikuchi, T., Aikawa, T., Kosaka, H., Pritchard, L., Ogura, N. & Jones, J.T. (2007). EST analysis of the pine wood nematode *Bursaphelenchus xylophilus* and *B. mucronatus*. *Molecular and Biochemical Parasitology* 155, 9-17.

Kikuchi, T., Li, H., Moens, M. & Jones, J. (2008). Identification of Expansin-like genes from the pine wood nematode *Bursaphelenchus xylophilus* and evolution of the expansin gene family within the Nematoda. *Molecular Plant Pathology,* (submitted).

Kikuchi, T., Shibuya, H., Aikawa, T. & Jones, J.T. (2006). Cloning and characterization of pectate lyases secreted by the pine wood nematode *Bursaphelenchus xylophilus*. *Molecular Plant-Microbe Interactions* 19, 280-287.

Kimber, M.J., McKinney, S., McMaster, S., Day, T. M., & Fleming, C.C. (2007). *flp* gene disruption in a parasitic nematode reveals motor dysfunction and unusual neuronal sensitivity to RNA interference. *The FASEB Journal* 21, 1233-1243.

Kishi, Y. (1995). *The pine wood nematode and the Japanese pine sawyer. Forests pests in Japan - No. 1*. Tokyo, Japan, Thomas Co. Ltd, 302 pp.

Kishi, Y. (1999). Influence of tree age on wilt and mortality of pines after inoculation with *Bursaphelenchus xylophilus*. *Journal of the Japanese Forestry Society* 81, 330-333.

Kiyohara, T. & Bolla, R.I. (1990). Pathogenic variability among populations of the pinewood nematode, *Bursaphelenchus xylophilus*. *Forest Science* 36, 1061-1076.

Kiyohara, T. & Tokushige, Y. (1971). Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. *Journal of the Japanese Forestry Society* 53, 210-218.

Kiyohara, T. (1973). Effect of temperature in the disease incidence of pine seedlings inoculated with *Bursaphelenchus lignicolus*. *Transactions of the meeting of the Japanese Forestry Society* 84, 334-335.

Kiyohara, T. (1976). The decrease of pathogenicity of pine wood nematode, Bursaphelenchus lignicolus, induced by the extended subculturing on the fungal mat of Botrytis cinerea. *Japanese Journal of Nematology* 6, 56-59.

Kiyohara, T., Kosaka, H., Aikawa, T., Ogura, N. & Tabata, K. (1999). Experiments of induced resistance to pine wilt disease in pine forest. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 103-104.

Kiyohara, T., Suzuki, K. & Hashimoto, H. (1975). Migration of the pine wood nematode in a pine tree at an early stage of infection. *Transactions of the meeting of the Japanese Forestry Society* 86, 299-300.

Knowles, K., Beaubien, Y., Wingfield, M.J., Baker, F.A. & French, D.W. (1983). The pinewood nematode new in Canada. *The Forestry Chronicle* 59, 40.

Kobayashi, F., Yamane, A. & Ikeda, T. (1984). The Japanese pine sawyer beetle as the vector of pine wilt disease. *Annual Review of Entomology* 29, 115-135.

Kobayashi, T., Sasaki, K. & Mamiya, Y. (1974). Fungi associated with *Bursaphelenchus lignicolus*, the pine wood nematode (I). *Journal of the Japanese Forestry Society* 56, 136-145.

Kobayashi, T., Sasaki, K. & Mamiya, Y. (1975). Fungi associated with *Bursaphelenchus lignicolus,* the pine wood nematode. (II). *Journal of the Japanese Forestry Society* 57, 184-193.

Kodan, A., Kuroda, H. & Sakai, F. (2002). A stilbene synthase from Japanese red pine (*Pinus densiflora*): Implications for phytoalexin accumulation and down-regulation of flavonoid biosynthesis. *Proceedings of the National Academy of Sciences of USA* 99, 3335-3339.

Kojima, K., Kamijyo, A., Masumori, M. & Sasaki, S. (1994). Cellulase activities of pine-wood nematode isolates with different virulences. *Journal of the Japanese Forestry Society* 76, 258-262.

Korenchenko, E.A. (1980). New species of nematodes from the family Aphelenchoididae, parasites of stem pests of the Dahurian Larch. *Zoologichesky Zhurnal* 59, 1768-1780.

Kosaka, H., Aikawa, T., Ogura, N., Tabata, K. & Kiyohara, T. (2001). Pine wilt disease caused by the pine wood nematode: the induced resistance of pine trees by the avirulent isolates of nematode. *European Journal of Plant Pathology* 107, 667-675.

Kruglik, I.A. & Eroshenko, A.S. (2004). *Bursaphelenchus fuchsi* sp. n. (Nematoda: Bursaphelenchidae): new nematode species from wood of pine *Pinus koraiensis*, Primorsky Territory. In: Sonin, M.D. (Ed.). *Paraziticheskie nematody rastenii i nasekomykh.* Moscow, Russia, Nauka, pp. 96-99.

Kudla, U., Qin, L., Milac, A., Kielac, A., Maissen, C., Overmars, H., Popeijus, H., Roze, E., Petrescu, A., Smant, G., Bakker, J. & Helder, J. (2005). Origin, distribution and 3D-modelling of Gr-EXPB1, an expansin from the potato cyst nematode *Globodera rostochiensis*. *FEBS Letters* 579, 2451-2457.

Kulinich, O.A., Kruglic, I.A., Eroshenko, A.S. & Kolosova, N.V. (1994). Occurrence and distribution of the nematode *Bursaphelenchus mucronatus* in the Russian Far East. *Russian Journal of Nematology* 2, 113-119.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 5, 150-163.

Kuroda, K. & Ito, S. (1992). Migration speed of pine wood nematodes and activities of other microbes during the development of pine-wilt disease in *Pinus thunbergii*. *Journal of the Japanese Forestry Society* 74, 383-389.

Kuroda, K. (1989). Terpenoids causing tracheid-cavitation in *Pinus thunbergii* infected by the pine wood nematode (*Bursaphelenchus xylophilus*). *Annals of the Phytopathological Society of Japan* 55, 170-178.

Kuroda, K. (1991). Mechanism of cavitation development in the pine wilt disease. *European Journal of Forest Pathology* 21, 82-89.

Kuroda, K., Yamada, T. & Ito, S. (1991). Development of the pine-wilt disease in *Pinus densiflora* from the standpoint of water conduction. *Journal of the Japanese Forestry Society* 73, 69-72.

Kusano, T., Nakamura, K., Fujii, T., Sugawara, Y., Ichikawa, Y., Masuyama, E., Shinkawa, H., Okamatsu, M., Kitaura, Y., Tsubaki, K., Uchida, T., Togashi, K., Enoki, S., Jikumaru, S., And Ikeda, S. (1999). RAPD-PCR fingerprinting patterns of six *Bursaphelenchus xylophilus* isolates and a *B. mucronatus* isolate. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 52-56.

Kusonoki, M. (1987). Symptom development of pine wilt disease – histopathological observations with electron microscopy. *Annals of the Phytopathological Society of Japan* 53, 622-629.

La, Y.J., Moon, Y.S., Yeo, W.H., Shin, S.C. & Bak, W.C. (1999). Recent status of pine wilt disease in Korea. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 239-241.

Lai, Y., Zhou, Y., Xu, Q. & Cheng, X. (2002). Controlling pine wilt disease with PP-bags + *Scleroderma* spp. *Journal of Jiangsu Forestry Science & Technology* 29, 18-20.

Laine, M.J., Haapalainen, M., Wahlroos, T., Kankare, K., Nissinen, R., Kassuwi, S. & Metzler, M.C. (2000). The cellulase encoded by the native plasmid of *Clavibacter michiganensis* ssp. *sepedonicus* plays a role in virulence and contains an expansin-like domain. *Physiology and Molecular Plant Pathology* 57, 221-233.

Lange, C., Burgermeister, W., Metge, K. & Braasch, H. (2007). Phylogenetic analysis of isolates of the *Bursaphelenchus sexdentati* group using ribosomal intergenic transcribed spacer DNA sequences. *Journal of Nematode Morphology and Systematics* 9, 95-108.

Lawler, C., Joyce, P. & Harmey, M.A. (1993). Immunological differentiation between *Bursaphelenchus xylophilus* and *B. mucronatus*. *Nematologica* 39, 536-546.

Leal, I., Green, M., Allen, E., Humble, L. & Rott, M. (2005). An effective PCR-based diagnostic method for the detection of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) in wood samples from lodgepole pine. *Nematology* 7, 833-842.

Leal, I., Green, M., Allen, E., Humble, L., & Rott, M. (2007). Application of a real-time PCR method for the detection of pine wood nematode, *Bursaphelenchus xylophilus*, in wood samples from lodgepole pine. *Nematology* 9, 351-362.

Lee, M.J. (1986). Resistance of pine species in Taiwan to pinewood nematode. *Quarterly Journal of Chinese Forestry* 19, 27-33.

Lee, S.M., Choo, H.Y., Park, N.C., Moon, Y.S. & Kim, J.B. (1990). Nematodes and insects associated with dead trees, and pine wood nematode detection in *Monochamus alternatus*. *Korean Journal of Applied Entomology* 29, 14-19.

Li, H., Shen, P., Fu, P., Lin, M. & Moens, M. (2007). Characteristics of the emergence of *Monochamus alternatus,* the vector of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), from *Pinus thunbergii* logs in Nanjing, China, and of the transmission of the nematodes through feeding wounds. *Nematology* 9, 807-816

Li, H., Trinh, P. Q., Waeyenberge, L. & Moens, M. (2008). *Bursaphelenchus chengi* sp. n. (Nematoda: Parasitaphelenchidae) isolated from packaging wood from Taiwan, in Nanjing (China). *Nematology* 10, 335-346.

Li, X. & Wang, Y. (1997). *Pinus strobus* var. *chiapensis* and other exotic pines: investigation and analysis of natural nematode infections and death. *Journal of Zhejiang Forestry College* 14, 273-276.

Li, Y., Li, F., Yu, D., Yang, W. & Zhang, H. (2003). *Bursaphelenchus xylophilus* was intercepted from imported wood packaging materials from Portugal in Shenzhen Port. *Plant Quarantine* 17, 110.

Li, Y., Yu, D., Li, F., Xie, W., Chen, J. & Zhang, Q. (2004). Molecular identification of *Bursaphelenchus xylophilus* by PCR amplification of first internal transcribed spacer of ribosomal DNA. *Plant Protection* 30, 61-63.

Liao, J., Zhang, L. & Feng, Z. (2001). Reliable identification of *Bursaphelenchus xylophilus* by rDNA amplification. *Nematologia Mediterranea* 29, 131-135.

Lin, M. & Zhou, M. (2004). Activity of avermectin for controlling the pine wood nematode, *Bursaphelenchus xylophilus*. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus*. Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1*. Leiden, The Netherlands, E. J. Brill, pp. 291.

Linit, M.J. (1988). Nematode-vector relationships in the pine wilt disease system. *Journal of Nematology* 20, 227-235.

Linit, M.J. (1990). Transmission of pinewood nematode through feeding wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). *Journal of Nematology* 22, 231-236.

Linit, M.J. & Tamura, M. (1987). Relative susceptibility of four pine species to infection by pinewood nematodes. *Journal of Nematology* 19, 44-50.

Linit, M.J., Kondo, E. & Smith, T. (1983). Insects associated with the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), in Missouri. *Environmental Entomology* 12, 467-470.

Liu, J. & Feng, Z. (1995). On the pathology of pine wilt disease caused by *Bursaphelenchus xylophilus*. *Acta Phytopathologica Sinica* 25, 171-174.

Liu, W., Yang, B, Xu, F., Ge, M., Zhang, P. & Wang, C. (1998). Study on early diagnosis for pine wilt disease caused by *Bursaphelenchus xylophilus*. I. Early diagnosis for pine wilt disease caused by *Bursaphelenchus xylophilus* parasitizing *Pinus massoniana* and *P. thunbergii*. *Forest Research* 11, 455-460.

Ma, Y. & Zhang, Y. (1997). The discovery of *Bursaphelenchus xylophilus* in wood packing case from Japan on Tianjin (Hebei) port. *Plant Quarantine* 11, 44.

Maeda, I., Kohara, Y., Yamamoto, M., & Sugimoto, A. (2001). Large scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. *Current Biology* 11, 171-176.

Malek, R.B. & Appleby, J.E. (1984). Epidemiology of pine wilt in Illinois. Disease distribution. *Plant Disease* 68, 180-186.

Mamiya, Y. (1972). Reproduction of pine lethal wilting disease by the inoculation of young trees with *Bursaphelenchus lignicolus*. *Japanese Journal of Nematology* 2, 40-44.

Mamiya, Y. (1980). Inoculation of the first year pine (*Pinus densiflora)* seedlings with the pine wood nematode *Bursaphelenchus lignicolus* and histopathology of diseased seedlings. *Japanese Journal of the Forestry Society* 62, 176-183.

Mamiya, Y. (1983). Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus*. *Annual review of Phytopathology* 21, 201-220.

Mamiya, Y. (1984)**.** The pine wood nematode. In: Nickle, W.R. (Ed.). *Plant and insect nematodes.* New York and Basel, Marcel Dekker, Inc., pp. 589-627.

Mamiya, Y. (1986). Interspecific hybridization between *Bursaphelenchus xylophilus* and *B. mucronatus* (Aphelenchida: Aphelenchoididae). *Applied Entomology and Zoology* 21, 159-163.

Mamiya, Y. (1988). History of pine wilt disease in Japan. *Journal of Nematology* 20, 219-226.

Mamiya, Y. (1999). Review on the pathogenicity of *Bursaphelenchus mucronatus.* In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 57-64.

Mamiya, Y. (2004). Pine wilt disease in Japan. In: Mota, M. and Vieira, P. (Eds). In: Mota, M. and Vieira, P. (Eds). *The pinewood nematode,* Bursaphelenchus xylophilus. *Nematology Monographs and Perspectives, vol. 1*. Leiden-Boston: Brill Academic Publishers, pp. 9-20.

Mamiya, Y. & Enda, N. (1972). Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). *Nematologica* 18, 159-162.

Mamiya, Y. & Enda, N. (1979). *Bursaphelenchus mucronatus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and its biology and pathogenicity to pine trees. *Nematologica* 25, 353-361.

Mamiya, Y. & Kiyohara, T. (1972). Description of *Bursaphelenchus lignicolus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and histopathology of nematode-infested trees. *Nematologica* 18, 120-124.

Mamiya, Y. Ikeda, T. & Shoji, T. (1989). Inoculation of the pine wood nematode, *Bursaphelenchus xylophilus* to *Pinus densiflora* shoot cuttings treated with benzoic acid. *Annals of the Phytopathological Society of Japan* 55, 303-308.

Matsunaga, K. & Togashi, K. (2004). A simple method for discriminating *Bursaphelenchus xylophilus* and *B. mucronatus* by species specific polymerase chain reaction primer pairs. *Nematology* 6, 273-277.

Matsuura, K. (1999). Analysis of preventive effects of systemic nematicides against pine wilt diseases. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 189-192.

McNamara, D.G. (2004). Quarantine concerns about the methods used to demonstrate pathogenicity of *Bursaphelenchus* spp. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus. *Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1.* Leiden, The Netherlands, E. J. Brill, pp. 187-197.

McNamara, D.G. & Stoen, M. (1988). A survey for *Bursaphelenchus* spp. in pine forests in Norway. *Bulletin OEPP* 18, 353-363.

Melakeberhan, H. & Webster, J.M. (1990). Relationship of *Bursaphelenchus xylophilus* population density to mortality of *Pinus sylvestris*. *Journal of Nematology* 22, 297-302.

Melakeberhan, H., Rutherford, T.A. & Webster, J.M. (1992). Influence of temperature on reproduction of *Bursaphelenchus xylophilus* and *Pinus sylvestris* mortality. *Nematologica* 38, 80-87.

Metge K. & Burgermeister W. (2006). Intraspecific variation in isolates of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) revealed by ISSR and RAPD fingerprints. *Journal of Plant Diseases and Protection* 113, 275-282.

Michalopoulos-Skarmoutsos, H., Skarmoutsos, G., Kalapanida, M., & Karageorgos, A. (2004). Surveying and recording of nematodes of the genus *Bursaphelenchus* in conifer forests in Greece and pathogenicity of the most important species. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus. *Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1.* Leiden, The Netherlands, E. J. Brill, pp. 113-126.

Mori, T. & Inoue, T. (1986). Pine-wood nematode-induced ethylene production in pine stems and cellulase as an inducer. *Journal of the Japanese Forestry Society* 68, 43-50.

Mota, M.M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K. & Sousa, E. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1, 727-734.

Myers, R.F. (1984).Comparative histology and pathology in conifers infected with pine wood nematode, Bursaphelenchus xylophilus. In Dropkin, V. ed. *Proceedings of the United States-Japan Seminar: The resistance mechanisms of pines against pine wilt disease, 7-11 May 1984, East-West Center, Honolulu, Hawaii.* Japan Society for the Promotion of Science, pp. 91-95.

Nakamura, K. & Yoshida, N. (2004). Successful control of pine wilt disease in Fukiage-hama seacoast pine forest in southwestern Japan. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus. *Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1.* Leiden, The Netherlands, E. J. Brill, pp. 269-281.

Nakamura, K., Togashi, K., Takahashi, F. & Futai, K. (1995). Different incidences of pine wilt disease in *Pinus densiflora* seedlings growing with different tree species. *Forest Science* 41, 841-850.

Nickle, W.R. (1970). A taxonomic review of the genera of the Aphelenchoidea (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). *Journal of Nematology* 2, 375-392.

Nickle, W.R., Golden, A.M., Mamiya, Y. & Wergin, W.P. (1981). On the taxonomy and morphology of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer 1934) Nickle 1970. *Journal of Nematology* 13, 385-392.

Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering*10, 1-6

Oda, K. (1967). The applicability of measurement of the oleoresin yield in determining the susceptibility of pine trees to beetle infestation. *Forest Pest News (Tokyo)* 16, 263-266.

Odani, K., Sasaki, S., Nishiyama, Y. & Yamamoto, N. (1985a). Early symptom development of the pine wilt disease by hydrolytic enzymes produced by the pine wood nematodes - cellulase as a possible candidate of the pathogen. *Journal of the Japanese Forestry Society* 67, 366-372.

Odani, K., Sasaki, S., Yamamoto, N., Nishiyama, Y. & Tamura, H. (1985b). Differences in dispersal of two associated nematodes, *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* in pine seedlings in relation to the pine wilt disease development. *Journal of the Japanese Forestry Society* 67, 398-403.

Ohba, K., Fukukoshi, T., Kurinobu, S. & Ishii, K. (1984). Susceptibility of subtropical pine species and provenances to the pine wood nematode. *Journal of the Japanese Forestry Society* 66, 465-468.

Oku, H. (1984). Biological activity of toxic metabolites isolated from pine trees naturally infected by pine wood nematodes. In Dropkin, V. (Ed). *Proceedings of the United States-Japan Seminar: The resistance mechanisms of pines against pine wilt disease, 7-11 May 1984, East-West Center, Honolulu, Hawaii.* Japan Society for the Promotion of Science Japan Society for the Promotion of Science, pp. 110-118.

Oku, H. (1988). Role of phytotoxins in pine wilt disease. *Journal of Nematology* 20, 245-251.

Oku, H. (1990). Phytotoxins in pine wilt disease [*Bursaphelenchus xylophilus*]. *Nippon Nogeikagaku Kaishi* 64, 1254-1257.

Oku, H., Shiraishi, T. & Kurozomi, S**.** (1979). Participation of toxin in wilting of Japanese pines caused by a nematode. *Naturwissenschaften* 67, 210.

Oku, H., Shiraishi, T., Ouchi, S., Kurozumi, S. & Ohta, H. (1980). Pine wilt toxin, the metabolite of a bacterium associated with a nematode. *Naturwissenschaften* 67, 198-199.

Palmisano, A.M., Ambrogioni, L., Tomiczek, C. & Brandstetter, M. (2004). *Bursaphelenchus sinensis* sp. n. and *B. thailandae* Braasch *et* Braasch-Bidasak in packaging wood from China. *Nematologia Mediterranea* 32, 57-65.

Panesar, T.S. & Sutherland, J.R. (1989). Pathogenicity of Canadian isolates of *Bursaphelenchus xylophilus* (pinewood nematode) to provenance of *Pinus sylvestris* and *P. contorta* in Finland. *Scandinavian Journal of Forest Research* 4, 549-557.

Peñas, A.C., Correia, P., Bravo, M.A., Mota, M. & Tenreiro, R. (2004). Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) associated with maritime pine in Portugal. *Nematology* 6, 437-453.

Peñas, A.C., Metge, K, Mota, M. & Valadas, V. (2006). *Bursaphelenchus antoniae* sp. n. (Nematoda: Parasitaphelenchidae) associated with *Hylobius* sp. from *Pinus pinaster* in Portugal. *Nematology* 8, 659-669.

Popeijus, H., Blok, V.C., Cardle, L., Bakker, E., Phillips, M.S., Helder, J., Smant, G., & Jones, J.T. (2000). Analysis of genes expressed in second stage juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* using the expressed sequence tag approach. *Nematology* 2, 567-574.

Qin, L., Kudla, U., Roze, E., Goverse, A., Popeijus, H., Nieuwland, J., Overmars, H., Jones, J.T., Schots, A., Smant, G., Bakker, J. & Helder, J. (2004). Identification of a functional expansin, a non-enzymatic, cell wall-loosening agent, from the plant parasitic nematode *Globodera rostochiensis*. *Nature* 427, 30.

Rautapää, J. (1986). Experiences with *Bursaphelenchus xylophilus* in Finland. *Bulletin OEPP* 16, 453-456.

Riga, E., Sutherland, J.R. & Webster, J.M. (1991). Pathogenicity of pinewood nematode isolates and hybrids to Scots pine seedlings. *Nematologica* 37, 285-292.

Robbins, K. (1982). Distribution of the pinewood nematode in the United States. In: Appleby, J.E. & Malek, R.B. (Eds). *Proceedings of the national pine wilt disease workshop. III. Natural History Survey, Champaign, IL*, USA, pp. 3-6.

Rodrigues, J. (2006). Eradication program for the pinewood nematode in Portugal. Pine wilt disease: a worldwide threat to forest ecosystems, International symposium. 10-14 July, Lisbon, 12 (Abst.).

Rosso, M.N., Dubrana, M.P., Cimbolini, N., Jaubert, S. & Abad, P. (2005). Application of RNA interference to root-knot nematode genes encoding oesophageal gland proteins. *Molecular Plant-Microbe Interactions* 18, 615-620.

Rosso, M.N., Favery, B., Piotte, C., Arthaud, L., De Boer, J.M., Hussey, R.S., Bakker, J., Baum, T.J. & Abad, P. (1999). Isolation of cDNA encoding a beta 1-4 endoglucanase in the root knot nematode *Meloidogyne incognita* and expression analysis during plant parasitism. *Molecular Plant-Microbe Interaction* 12, 585-591.

Roze, E., Overmars, H., Mitreva, M., Bakker, J. & Smant, G. (2008). Identification and characterization of expansin-like proteins from the root-knot nematode *Meloidogyne chitwoodi*. *Molecular Plant-Microbe Interaction* (submitted).

Rutherford, T.A., Mamiya,Y. & Webster, J.M. (1990). Nematode-induced pine wilt disease: factors influencing its occurrence and distribution. *Forest Science* 36, 145-155.

Ryss, A., Vieira, P., Mota, M. & Kulinich, O. (2005). A synopsis of the genus *Bursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species. *Nematology* 7, 393-458.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

Saloheimo, M., Paloheimo, M., Hakola, S., Pere, J., Swanson, B., Nyyssonen, E., Bhatia, A., Ward, M. & Penttila, M. (2002). Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. *European Journal of Biochemistry* 269, 4202-4211.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). Molecular cloning. A laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.

Schauer-Blume, M. (1990). Preliminary investigations on pathogenicity of European *Bursaphelenchus* spp. in comparison to *Bursaphelenchus xylophilus* from Japan. *Revue de Nématologie* 13, 191-196.

Scholl, E.H., Thorne, J.L., McCarter, J.P. & Bird, D.M. (2003). Horizontally transferred genes in plant-parasitic nematodes: a high-throughput genomic approach. *Genome Biology* 4, no.-R39.

Schönfeld, U., Braasch, H. & Burgermeister W. (2006). *Bursaphelenchus* spp. (Nematoda: Parasitaphelenchidae) in wood chips from sawmills in Brandenburg and description of *Bursaphelenchus willibaldi* sp. n.. *Russian Journal of Nematology* 14, 119-126.

Seinhorst, J.W. (1959). A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica* 4, 67-69.

Shaheen, F., Winter, R.E.K. & Bolla, R.I. (1984). Phytotoxin production in *Bursaphelenchus xylophilus*-infected *Pinus sylvestris*. *Journal of Nematology* 16, 57-61.

Shen, P., Jiao, G. & Li, H. (1995). Pathogenicity differences between pine wood nematode (*Bursaphelenchus xylophilus*) from Nanjing, China and Japan. *Forest Diseases and Pests Newsletter* 4, 1-2.

Shibata, E. & Okuda, K. (1989). Transmission of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner *et* Buhrer) Nickle (Nematoda: Aphelenchoididae), by the Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), to pine twigs under laboratory conditions, *Japanese Journal of Nematology* 18, 6-14.

Shimazu, M. (2004). Effects of temperature on growth of *Beauveria bassiana* F-263, a strain highly virulent to the Japanese pine sawyer, *Monochamus alternatus*, especially tolerance to high temperatures. *Applied Entomology and Zoology* 39, 469-475.

Shimazu, M. (2006). Current status on research and management of pine wilt disease in Japan. *Current status on research and management of pine wilt disease, International Symposium, October 20*. Korea Forest Research Institute, Seoul, Korea, pp. 1-18.

Shimazu, M., Tsuchiya, D., Sato, H. & Kushida, T. (1995). Microbial control of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) by application of nonwoven fabric strips with *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) on infested tree trunks. *Applied Entomology and Zoology* 30, 207-213.

Shimizu, I., Suzuki, K. & Fukuda, K. (1988). Effect of site conditions on the development of pine wilt disease. Gap effect. *Bulletin of the Tokyo University Forests*, 95-101.

Shin, S. & Han, H. (2006). Current status on research and management of pine wilt disease in Korea. *Current status on research and management of pine wilt disease, International Symposium, October 20.* Korea Forest Research Institute, Seoul, Korea, pp. 31-44.

Sikora, E.J. & Malek, R.B. (1991). Influence of temperature on development of pine wilt in Scots pine. *Journal of Nematology* 23, 188-193.

Skarmoutsos, G. & Michalopoulos-Skarmoutsos, H. (2000). Pathogenicity of *Bursaphelenchus sexdentati, Bursaphelenchus leoni* and *Bursaphelenchus hellenicus* on European pine seedlings. *Forest Pathology* 30, 149-156.

Skarmoutsos, G. & Skarmoutsos, H. (1999). First record of *Bursaphelenchus* nematodes from pine forests in Greece. *Plant Disease* 83, 879.

Skarmoutsos, G., Braasch, H. & Michalopoulou, H. (1998). *Bursaphelenchus hellenicus* sp. n. (Nematoda: Aphelenchoididae) from Greek pine wood. *Nematologica* 44, 623-629.

Smant, G., Stokkermans, J.P.W.G., Yan, Y., De Boer, J.M., Baum, T., Wang, X., Hussey, R.S., Gommers, F.J., Henrissat, B., Davis, E.L., Helder, J., Schots, A. & Bakker, J. (1998). Endogenous cellulases in animals: Isolation of β-1,4-endoglucanase genes from two species of plant parasitic nematodes. *Proceedings of the National Academy of Sciences of USA* 95, 4906-4911.

Sousa, E., Bravo, M.A., Pires, J., Naves, P., Peñas, A.C., Bonifacio, L. & Mota, M.M. (2001). *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) associates with *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal. *Nematology* 3, 89-91.

Southey, J.F. (1986). Laboratory methods for work with plant and soil nematodes. M.A.F.F.publication, 9th edn London: HMSO, 202 pp.

Steiner, G. & Buhrer, E.M. (1934). *Aphelenchoides xylophilus* n. sp., a nematode associated with blue-stain and other fungi in timber. *Journal of Agricultural Research* 48, 949- 951.

Sutherland, J. & Peterson, M. (1999). The pinewood nematode in Canada: history, distribution, hosts, potential vectors and research. In: Futai, K., Togashi, K. & Ikeda, T. (Eds*). Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998.* Kyoto, Japan, Shokado Shoten, pp. 247-253.

Sutherland, J.R., Ring, F.M. & Seed, J.E. (1991). Canadian conifers as hosts of the pinewood nematode (*Bursaphelenchus xylophilus*): results of seedling inoculations. *Scandinavian Journal of Forest Research* 6, 209-216.

Suzuki, K. (1984). General effect of water stress on the development of pine wilting disease caused by *Bursaphelenchus xylophilus*. *Bulletin Forestry and Forest Products Research Institute, Japan* 325, 97-126.

Suzuki, K. (2002). Pine wilt disease – a threat to pine forest in Europe. *Dendrobiology* 48, 71-74.

Suzuki, K. (2004). Pine wilt disease – a threat to pine forests in Europe. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus*. Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1.* Leiden, The Netherlands, E. J. Brill, pp. 25-30.

Suzuki, K. & Kiyohara, T. (1978). Incidence of water stress on development of pine wilting disease caused by *Bursaphelenchus lignicolus*. *European Journal of Forest Pathology* 8, 97-107.

Swofford. D.L. (2002). *PAUP, phylogenetic analysis using parsimony and other methods. Version 4.* Sunderland, Massachusetts, Sinauer Associates, 128 pp.

Tada, M., Omizu, A. & Shiroishi, M. (1981). Effect of phosphate on the growth and toxin-producing ability of a bacterium isolated from pine wood nematode *Bursaphelenchus lignicolus. The science reports of Faculty of Agriculture, Okayama University, Japan* 57, 35-40

Takasu, F., Yamamoto, N., Kawasaki, K., Togashi, K., Kishi, Y. & Shigesada, N. (2000). Modelling the expansion of an introduced tree disease. *Biological Invasions* 2, 141-150.

Takeuchi, Y., Kanzaki, N. & Futai, K. (2005). A nested PCR-based method for detecting the pine wood nematode, *Bursaphelenchus xylophilus*, from pine wood. *Nematology* 7, 775-782.

Tamura, H. & Dropkin, V. (1984). Resistance of pine trees to pine wilt caused by the nematode *Bursaphelenchus xylophilus*. *Journal of the Japanese Forestry Society* 66, 306-312.

Taniwaki, T., Okitsu, M., & Kishi, Y. (2004). Diurnal emergence of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) from pine logs. *Journal of the Japanese Forestry Society* 86, 158-163.

Tarès, S., Abad, P., Bruguier, N. & Guiran, G.D. (1992). Identification and evidence for relationships among geographical isolates of *Bursaphelenchus* spp. (pinewood nematode) using homologous DNA probes. *Heredity* 68, 157-164.

Tarès, S., Lemontey, J.M., Guiran, G., & Abad, P. (1993). Cloning and characterization of a highly conserved satellite DNA sequence specific for the phytoparasitic nematode *Bursaphelenchus xylophilus*. *Gene* 129, 269-273.

Tarès, S., Lemontey, J.M., Guiran, G. & Abad, P. (1994). Use of species-specific satellite DNA from *Bursaphelenchus xylophilus* as a diagnostic probe. *Phytopathology* 84, 294-298.

Tarjan, A. & Baéza-Aragon, C. (1982). An analyses of the genus *Bursaphelenchus* Fuchs, 1937. *Nematropica* 12, 121-135.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997). The ClustalX windows inference: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research* 24, 4876-4882.

Toda, T. (2004). Studies on the breeding for resistance to the pine wilt disease in *Pinus densiflora* and *P. thunbergii*. *Bulletin of the Forest Tree Breeding Center* 20, 83-217.

Toda, T., Kurinobu, S. & Sasaki, M. (2002). Growth and survival rate at the seventh year after selecting the progenies of pine trees for resistance to pine wood nematodes by inoculation test. J*ournal of the Japanese Forestry Society* 84, 188-192.

Togashi, K. (1985). Transmission curves of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) from its vector, *Monochamus alternatus* (Coleoptera: Cerambycidae), to pine trees with reference to population performance. *Applied Entomology and Zoology* 20, 246-251.

Togashi, K. (1988). Population density of M*onochamus alternatus* adults (Coleoptera: Cerambycidae) and incidence of pine wilt disease caused by *Bursaphelenchus xylophilus (*Nematoda: Aphelenchoididae). *Researches on Population Ecology* 30, 177-192.

Togashi, K. (1989a). Development of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) in *Pinus thunbergii* trees weakened at different times. *Journal of the Japanese Forestry Society* 71, 383-386.

Togashi, K. (1989b). Factors affecting the number of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) carried by newly emerged adults of *Monochamus alternatus* (Coleoptera: Cerambycidae). *Applied Entomology and Zoology* 24, 379-386.

Togashi, K. & Sekizuka, H. (1982). Influence of the pine wood nematode, *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae), on longevity of its vector, *Monochamus alternatus* (Coleoptera: Cerambycidae). *Applied Entomology and Zoology* 17, 160-165.

Tokushige, Y. & T. Kiyohara (1969). *Bursaphelenchus* sp. in the wood of dead pine trees. *Journal of the Japanese Forestry Society* 51, 193-195.

Tomiczek, C., Braasch, H., Burgermeister, W., Metge, K., Hoyer, U. & Brandstetter, M. (2003). Identification of *Bursaphelenchus* spp. isolated from Chinese packaging wood imported to Austria. *Nematology* 5, 573-581.

Tomminen, J., Nuorteva, M., Pulkkinen, M. & Vokevo, J. (1989). Occurrence of the nematode *Bursaphelenchus mucronatus* Mamiya & Enda, 1979 (Nematoda: Aphelenchoididae) in Finland. *Silva Fennica* 23, 271-277.

Trudgill, D.L. (1991). Resistance to and tolerance of plant parasitic nematodes in plants. *Annual Review of Phytopathology* 29, 167-192.

Tzean, S. & Jan, S. (1985a). The occurrence of pinewood nematode, *Bursaphelenchus xylophilus*, in Taiwan. *Proceedings of the 6th ROC symposium of electron microscopy*, pp. 38-39. [Abstr.]

Tzean, S. & Jan, S. (1985b). Pine wilt disease caused by pinewood nematode (*Bursaphelenchus xylophilus*) and its occurrence in Taiwan. *Phytopathologist and Entomologist* 12, 1-19.

Urwin, P.E., Lilley, C.J. & Atkinson, H.J. (2002). Ingestion of double stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. *Molecular Plant-Microbe Interactions* 15, 747-752.

Vieira, P., Burgermeister, W., Mota, M., Metge, K. & Silva, G. (2007). Lack of genetic variation of *Bursaphelenchus xylophilus* in Portugal revealed by RAPD-PCR analyses. *Journal of Nematology* 39, 118-126.

Vrain, T.C. (1993). Restriction fragment length polymorphism separates species of the *Xiphinema americanum* group. *Journal of Nematology* 25, 361-364.

Walia, K.K., Negi, S., Bajaj, H.K. & Kalia, D.C. (2003). Two new species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae) from pine wood and insect frass from India. *Indian Journal of Nematology* 33, 1-5.

Wang, H., Yang, Z. & Zhang, S. (2005). *Bursaphelenchus rainulfi* Braasch, 2002, a new recorded species in Japan and its identification. *Journal of Fujian Agriculture and Forestry University (Natural Science Edition)* 34, 158-161.

Wang, L., Wang, Y., Yang, B., Hu, X. & Yu, S. (2001). Studies on relationships of *Bursaphelenchus xylophilus* and *B. mucronatus* by RAPD. A*cta Phytopathologica Sinica* 31, 225-229.

Wang, Q., Xu, F., Ge, M., Wang, Z. & Chen, T. (1997). A preliminary study on the variation of resistance in 39 Masson pine provenances. *Journal of the Zhejiang Forestry College* 14, 29-34.

Warren, J.E. & Linit, M.J. (1992). Within-wood spatial dispersion of the pinewood nematode, *Bursaphelenchus xylophilus*. J*ournal of Nematology* 24, 489-494.

Webster, J.M. (1999). Pine wilt disease: a world wide survey. Sustainability of pine forests in relation to pine wilt and decline. *Proceedings of International Symposium, Tokyo, Japan,* 27-28 October, 1998. pp. 254-260.

Webster, J.M (2004). The pine wood nematode: implications of factors past and present for pine wilt disease. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus*. Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1*. Leiden, The Netherlands, E. J. Brill, pp. 55-64.

Webster, J.M., Anderson, R.V., Baillie, D.L., Beckenbach, K., Curran, J. & Rutherford, T.A. (1990). DNA probes for differentiating isolates of the pinewood nematode species complex. *Revue de Nématologie* 13, 255-263.

Wingfield, M.J. (1983). Transmission of pine wood nematode to cut timber and girdled trees. *Plant Disease* 67, 35-37.

Wingfield, M.J. &. Blanchette, R.A. (1983). Transmission of the pine wood nematode, *Bursaphelenchus xylophilus* during oviposition of *Monochamus carolinensis*, *M. scutellatus* and *Monochamus mutator*. *Phytophatology* 73, 839-839.

Wingfield, M., Blanchette, A. & Kondo, E. (1983). Comparison of the pine wood nematode, *Bursaphelenchus xylophilus* from pine and balsam fir. *European Journal of Forest Pathology* 13, 360-373.

Wingfield, M., Bedker, P. & Blanchette, R. (1984). Observations on field grown pines inoculated with the pine wood nematode. *Phytopathology* 74, 841.

Xie, L., Zhao, B., Ju, Y. & Liang, B. (2002). Observation on the bacteria carried by pine wood nematode through optical microscope and its number measurement. *Journal of Zhejiang Forestry College* 19, 346-349.

Xu, F., Xi, K., Gang, X., Zhou, Y., Xu, W., Xu, K. & Ge, M. (1994a). Study on the resistances of various year classes of *Pinus massoniana* to pine wood nematode (PWN), *Bursaphelenchus xylophilus*. *Journal of Nanjing Forestry University 1*8, 27-34.

Xu, F., Xi, K., Yan, B., Ge, M. & Sun, Y. (1994b). Studies on the emergence, replenishing feeding methods of adults of *Monochamus alternatus* in the area of Nanjing and its control. *Forest Research* 7, 215-219.

Xu, F., Ge, M., Zhu, Z. & Zhu, K. (1996). Studies on resistance of pine species and Masson pine provenances to *Bursaphelenchus xylophilus* and the epidemic characteristics of the nematode in Nanjing. *Forest Research* 9, 521-524.

Xu, F., Ge, M., Wang, Q., Zhang, P., Zhu, K., Zhao, J., Xu, D. & Wang, M. (1998). Studies on masson pine provenances resistance to pine wood nematode (PWN) disease in China. *Journal of Nanjing Forestry University* 22, 29-33.

Xu, K., Xu, F., Wang, M., Zhao, J. Jiang, Q., Zhang, P., Xu, D., He, R. & Jiang, X. (2002). The techniques of *Scleroderma guani* Xiao et Wu to control pine sawyer beetles. *Journal of Nanjing Forestry University* 26, 48-52.

Xu, P., Shen, P., Tai, M. & Jiao, G. (1995). The discovery of *Bursaphelenchus xylophilus* on imported wooden packing. *Plant Quarantine* 9, 297-298.

Xu, Y., Gu, J.F., Zhang, J.C., Cui, J.X. & Zhang, Y.F. (2006). Identification of *Bursaphelenchus rainulfi* in *Pinus massoniana* in Ningbo. *Journal of Zhejiang Forestry Science and Technology* 26, 37-39.

Yadav, B.C., Veluthambi, K. & Subramaniam, K. (2006). Host-generated double-stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. *Molecular and Biochemical Parasitology* 148, 219-222.

Yamamoto, N., Odani, K., Sasaki, S. & Nishiyama, Y. (1986). Cellulase exudation by the pine wood nematode - detection of activity in its crawling track. *Journal of the Japanese Forestry Society* 68, 237-240.

Yamane, A., Miyoshi, M., Hatta, Y., Nakamura, T., Iwata, R., Ishikawa, T., Kobayashi, H. & Mamiya, Y. (2004). Pine wood nematode movement on, and migration from, the body surface of the Japanese pine sawyer during feeding on pine twigs after emergence. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode*, Bursaphelenchus xylophilus. *Proceedings of an International Workshop, University of Évora, Portugal, August 20-22, 2001. Nematology monographs and perspectives, Volume 1*. Leiden, The Netherlands, E. J. Brill, pp. 247-260.

Yan, D. & Yang, B. (1997). The enzymes in the secretions of pine wood nematode (*Bursaphelenchus xylophilus*). *Forest Research* 10, 265-269.

Yan, Y., Smant, G., Stokkermans, J., Qin, L., Helder, J., Baum, T., Schots, A. & Davis, E. (1998). Genomic organization of four beta-1,4-endoglucanase genes in plant-parasitic cyst nematodes and its evolutionary implications. *Gene* 220, 61-70.

Yang, B. (2002). Advance in research of pathogenic mechanism of pine wood nematode. *Forest Pest and Disease*, 21, 27-31.

Yang, B. (2004). The history, dispersal and potential threat of pine wood nematode in China. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematodes,* Bursaphelenchus xylophilus: *proceedings of an international workshop, University of Évora, Portugal, August 20-22, 2001*. Nematology Monographs and Perspectives, Volume 1, 21-24.

Yang, B. & Wang, Q. (1989). Distribution of the pinewood nematode in China and susceptibility of some Chinese and exotic pines to the nematode. *Canadian Journal of Forest Research* 19, 1527-1530.

Yang, B., Wang, Q. & Zhou, W. (1987). The resistance of pine species to pine wood nematode, *Bursaphelenchus xylophilus*. *Acta Phytopathologica Sinica* 17, 211-214.

Yang, B., Wang, Q., Zou, W. & Li, Y. (1988). Study on the pathogenicity of *Bursaphelenchus mucronatus* to pines. *Forest Science and Technology* 1, 21-23.

Yang, B., Liu, W., Xu, F. & Zhang, P. (1999). The potential threat of pine wilt disease to China forest and its early diagnosis. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 261-265.

Yang, B., Wang, L., Zhao, W., Xu, F., Zhang, P. & Li, Z. (2002). The latent infection of *Bursaphelenchus xylophilus a*nd a new transmission way of PWN by *Monochamus alternatus. Forest Research, Beijing* 15, 251-255.

Yano, M. (1913). Investigation on the cause of pine mortality in Nagasaki Prefecture. *Sanrinkoho* 4, 1-14.

Ye, W., Giblin-Davis, R.M., Braasch, H., Morris, K. & Thomas W.K. (2007). Phylogenetic relationships among *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution* 43, 1185-1197.

Yi, C., Byun, B., Park, J., Yang, S. & Chang, K. (1989). First finding of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner *et* Buhrer) Nickle and its insect vector in Korea. *Research Reports of the Forestry Research Institute Seoul* 38, 141-149.

Yin, K., Fang, Y. & Tarjan, A.C. (1988). A key to species in the genus *Bursaphelenchus* with a description of *Bursaphelenchus hunanensis* sp. n. (Nematoda: Aphelenchoididae) found in pine wood in Hunan Province, China. *Proceedings of the Helminthological Society of Washington* 55, 1-11.

Zhang, H., Kanzaki, H. & Kawazu, K. (1997). Benzoic acid accumulation in the Pinus thunbergii callus inoculated with the pine wood nematode, *Bursaphelenchus xylophilus*. *Zeitschrift für Naturforschung. Section C, Biosciences* 52, 329-332.

Zhang, K., Lin, M., Wen, L. & Xu, W. (1999). Genetic variation of *Bursaphelenchus xylophilus* and *B. mucronatus* geographical isolates of China as shown by RAPD's. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 65-69.

Zhang, L., Kong, F. & Yang, B. (2002) Intra and interspecific variation in *Bursaphelenchus xylophilus* and *B. mucronatus* revealed by mtDNA polymorphism. *Forest Research* 15, 7-12.

Zhang, L., Liao, J. & Feng, Z. (2001). Sequencing and PCR-SSCP analysis of ribosomal DNA of *Bursaphelenchus* nematodes. *Acta Phytopathologica Sinica* 31, 84-89.

Zhang, Q., Bai, G., Yang, W., Li, H. & Xiong, H. (2006). Pathogenic cellulase assay of pine wilt disease and immunological localisation. *Bioscience Biotechnology Biochemistry* 70, 2727-2732.

Zhang, X., Stamps, W.T. & Linit, M.J. (1995). A non-destructive method of determining *Bursaphelenchus xylophilus* infestation of *Monochamus* spp. vectors. *Journal of Nematology* 27, 36-41.

Zhao, B. & Lin, F. (2005). Mutualistic symbiosis between *Bursaphelenchus xylophilus* and bacteria of the genus *Pseudomonas*. *Forest Pathology* 35, 339-345.

Zhao, B., Guo, D.S., Gao, R. & Guo, J. (2000a). A preliminary study on the relationship between the bacterial isolate B619 and pine wilt disease. *Journal of Nanjing Forestry University* 24, 72-74.

Zhao, B., Guo, D. & Gao, R. (2000b). Observation of the site of pine wood nematode where bacteria are carried with SEM and TEM. *Journal of Nanjing Forestry University* 24, 69-71.

Zhao, B., Guo, D., Gao, R. & Guo, J. (2000c). A preliminary study on the relationship between the bacterial isolate B619 and pine wilt disease. *Journal of Nanjing Forestry University* 24, 72-74.

Zhao, B., Liu Y. & Lin, F**.** (2007). Effects of bacteria associated with pine wood nematode (*Bursaphelenchus xylophilus*) on development and egg production of the nematode. *Journal of Phytopathology* 155, 26-30.

Zhao, B., Wang, H.L., Han, S.F. & Han, Z.M. (2003). Distribution and pathogenicity of bacteria species carried by *Bursaphelenchus xylophilus* in China. *Nematology* 5, 899-906.

Zhao, J., Zhang C., Dai, J, Jiang, P., Sun, H. & Zhou G. (1999). Studies of emerge emigration of *Monochamus alternatus* and its ability of carrying nematode. *Forest Research* 12, 572-576.

Zhao, J., Jiang, P., Wu, C., Sun, S., Jian, L. & Lin, C. (2000). Studies on *Monochamus alternatus* attractant and the attractability. *Forest Research* 13, 262-267.

Zhao, J., Yu, S., Yao, J., Lin, C., Ding, D. & Wang, H. (2004). PWN risk assessment in Huangshan Scenic Área II. Monitoring of nematode carried by pine borer beetles. *Forest Research* 17, 72-76.

Zhao, Z., Li, D., Hu, X., Xu, F., Sun, Z., Hu, G. & Liu, X. (1999). Study on variations of neutral terpenoids of resistant provenances of *P. massoniana* after inoculating *Bursaphelenchus xylophilus*. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline. Proceedings of the Symposium, Tokyo, Japan, 26-30 October 1998*. Kyoto, Japan, Shokado Shoten, pp. 217-221.

Zheng, J., Subbotin, S.A., He, S., Gu, J. & Moens, M. (2003). Molecular characterisation of some Asian isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* using PCR-RFLPs and sequences of ribosomal DNA. *Russian Journal of Nematology* 11, 17-22.

Zheng, J., Xu, J., Wu, Y. & Li, D. (1998). RAPD fingerprinting on inter- and infra-species of *Bursaphelenchus xylophilus* and *B. mucronatus*. *Journal of Zhejiang Agricultural University* 24, 597-601

Zhuo, K., Li., X., Li, D., Yu, S. & Liao, J. (2007). *Bursaphelenchus uncispicularis* n. sp. (Nematoda: Parasitaphelenchidae) from *Pinus yunnanensis* in China. *Nematology* 9, 237-242.

Curriculum Vitae

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Education

2003-2007-: PhD student at Ghent University, Faculty of Bioscience Engineering and Institute for Agricultural and Fisheries Research, Merelbeke (Belgium).

2000-2001: Master of Science (MSc) in Nematology with great distinction, Department of Biology, Faculty of Science, Ghent University, Belgium. Thesis title "Screening microsatellites from *Xiphinema index* (Dorylaimida: Longidoridea)" under supervision of Prof. Dr ir. Maurice Moens.

1991-1994: Master of Science (MSc) in Plant Pathology (Nematology), Nanjing Agricultural University, China. Thesis title "Studies on parasitic nematodes of fig (*Ficus carica*) in Jiangsu province, China" under supervision of Prof. Dr Hurui Cheng, Department of Plant Pathology, Nanjing Agricultural University.

1987-1991: Bachelor of Agronomy (BA) in Plant Pathology, Nanjing Agricultural University, China,

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From September 1994 to May 2003, lecturer in the Department of Plant Pathology, Nanjing Agricultural University.

From May 2003 to now, associate professor in the Department of Plant Pathology, Nanjing Agricultural University.

Publications

Li, H., Trinh, P. Q., Waeyenberge, L. & Moens, M. (2008). *Bursaphelenchus chengi* sp. n. (Nematoda: Parasitaphelenchidae) isolated at Nanjing, China, in packaging wood from Taiwan. *Nematology* 10, 335-346.

Li, H., Shen, P., Fu, P., Lin, M. & Moens, M. (2007). Characteristics of the emergence of *Monochamus alternatus*, the vector of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), from *Pinus thunbergii* logs in Nanjing, China, and of the transmission of the nematodes through feeding wounds. *Nematology* 9, 807-816.

Li, H., Trinh, P. Q., Waeyenberge, L. & Moens, M. (2008). Morphological, morphometrical and molecular characterization of *Bursaphelenchus* spp. isolated from imported packaging wood in Nanjing, China. *Nematology* (submitted).

Jones, J., Moens, M., Mota, M., **Li, H.** & Kikuchi, T. (2008). *Bursaphelenchus xylophilus*: opportunities in comparative genomics and molecular host-parasite interactions. *Molecular Plant Pathology* 9, online available.

Kikuchi, T., **Li, H.**, Moens, M. & Jones, J. (2008). Identification of Expansin-like genes from the pine wood nematode *Bursaphelenchus xylophilus* and evolution of the expansin gene family within the Nematoda. (submitted).

Wang, X., **Li, H.**, Hu, Y., Fu. P. & Xu. J. (2007). Molecular cloning and analysis of a new venom allergen-like protein gene from the root-knot nematode *Meloidogyne incognita. Experimental Parasitology* 117, 133-140.

Wang, X., **Li, H.**, Hu, Y. & Xu, J. (2007). *Meloidogyne hispanica* Hirschmann, 1986, a new record species of root-knot nematode in China. *Acta Phytopathologica Sinica* 37, 321-324.

Hu. Y., Wang, X. & **Li, H.** (2007) Molecular characterization and the diagnosis of *Meloidogyne hispanica. Journal of Nanjing Agricultural University* 30, 62-65.

Liu, W., Lin, M., **Li, H.** & Sun, M. (2007). Study on dynamic development of *Aphelenchoides besseyi* on rice plant by artificial inoculations in greenhouse. *Scientia Agricultura Sinica* 40, 2734-2740.

Ding, G., Fu, P., **Li, H.** & Guo, J. (2005). Biocontrol evaluation of *Bacillus subtilus* strain AR11 against *Meloidogyne incognita*. *Journal of Nanjing Agricultural University* 28, 46-49.

Shen, P., Li, M., **Li, H.** & Moens, M. (2005). A key to species of the genus *Bursaphelenchus*. *Plant Quarantine* 2(19), 76-83.

Fu, P., **Li, H.**, Ding, G. & Guo, J. (2004) Biocontrol evaluation of strain GJ23 against *Meloidogyne incognita. Journal of Jiangsu Agricultural Sciences* 5, 43-45.

Shen, P. **Li, H.** He, Y. & Moens, M. (2004). Isolation of microsatellites for *Xiphinema americanum* and their use in taxonomy. *Science of Inspection and Quarantine,* 4(14), 16-22.

He, Y., **Li, H.**, Brown, D.J.F., Lamberti, F. & Moens, M. (2003). Isolation and characterisation of microsatellites for *Xiphinema index* using degenerate oligonucleotide primed PCR. *Nematology* 5: 809-819.

Hong, X., Xu, H., **Li, H.** & Xie, L. (2003). Alien invasive insects and pathogens in Jiangsu Province: current status, influence and control. *Journal of Nanjing Agricultural University* 6, 116-123.

Xu, J., Narabu, T., **Li, H.** & Fu, P. (2002). Preparation of *Meloidogyne javanica* near-isogenic lines virulent and avirulent against the tomato resistance gene Mi and preliminary analyses of the genetic variation between the two lines. *Acta Genetic Sinica* 29, 212-216.

Li, H., Fu. P., Shen, P. & Xu, J. (2001). Studies on quarantine techniques for parasitic nematodes in export garden plants. *Journal of Nanjing Agricultural University* 24, 31-34.

Li, H., Shen, P. & Xu, J. (2000). Investigations on parasitic nematodes of import and export garden plants in Jiangsu province. *Journal of Nanjing Agricultural University* 23, 34-38.

Shen, P., **Li, H.** & Xu, J. (2001). Investigation on parasite nematodes of import and export garden plants in Jiangsu privies and treatment technique in quarantine. *Plant Quarantine*, 2(15), 68-73.

Li, H., Xu, J. & Shen, P. (1999). Distribution and seasonal dynamic changes of nematode parasites in fig main growing areas in Jiangsu province. *Journal of Nanjing Agricultural University* 22, 38-41.

Xu, J., **Li, H.** & Shen, P. (1999). Biological test of pathogenic variation among populations of *Meloidogyne incognita* from China. *Journal of Nanjing Agricultural University* 22, 33-36.

Shen, P., **Li, H.** & Gu, Z. (1999). *Xiphinema krugi* found in China associated with import potted landscape. *Plant Quarantine*, 13(2), 69-73.

Shen, P., **Li, H.** & Xu, J. (1998). Identification of stem nematodes from import and export plant and studies on their pathogenicities. *Journal of China Agricultural University*, 3(*Supl*.), 88-92.

Jiao, G., Shen, P. & **Li, H.** (1996). Studies on pathogenicity of pine wood nematodes from Japan and Nanjing, China to *Cedrus deodara* and *Pinus massoniana. Plant Quarantine*, 10(4), 193-195.

Shen, P., Jiao, G. & **Li, H.** (1995). Pathogenicity differences between pine wood nematode (*Bursaphelenchus xylophilus*) from Nanjing, China and Japan. *Forest diseases and Pests Newsletter* 4, 1-2.

Shen, P., **Li, H.** & Chen, P. (1994). *Anguina agrostis*. *Plant Quarantine*, 8(6), 349-352.

Symposium

Li, H. & Moens, M. (2007), The emergence of *Monochamus alternatus* from *Pinus thunbergii* logs and the transmission of *Bursaphelenchus xylophilus* through vector feeding wounds in Nanjing, China. *Advances in Nematology*, Association of Applied Biologists, Linnean Society, London, UK, 11 December, 2007 (Oral presentation).

Awards

Participated project "Studies on Longidoridae and Trichodoridae in China" was awarded the third degree prize of Ministry of Education in China (1998).

Participated project "Studies on Pine Wood Nematodes (*Bursaphelenchus xylophilus*) of Chinese Populations and Japanese Populations Intercepted from Ports" was awarded the third degree prize of Ministry of Agriculture in China. (1998).

Teaching

Courses offered in Chinese for undergraduate and graduate students:

1. General Plant Pathology
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3. Horticultural Plant Diseases
4. Plant Nematology